Ovine listeriosis: development of novel serological assays for diagnosis, experimental validation and field investigations into the epidemiology of infection.

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Declaration.

I declare that this thesis has been composed by myself and the work described is entirely my own except where clearly stated.

J.C. Low.

January 1993.

Dedication.

This work is dedicated to Kay, Emma and Robert.

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 3.5.10)

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Title of Thesis OVINE LISTERIOSIS: DEVELOPMENT OF NOVEL SEROLOGICAL ASSAYS FOR DIAGNOSIS, EXPERIMENTAL VALIDATION AND FIELD INVESTIGATIONS INTO THE EPIDEMIOLOGY OF INFECTION.

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A panel of anti-*Listeria* monoclonal antibodies (mAbs) were produced following the immunization of mice with heat-killed *Listeria monocytogenes* serovar 4b. Five of the mAbs recognised an antigen irregularly present in a number of species and serovars of the genera *Listeria* and *Bacillus*. The antigen spontaneously adhered to sheep erythrocytes and was putatively identified as lipoteichoic acid (LTA). A competitive sandwich ELISA was developed which revealed that three of the mAbs recognised the same or overlapping antigenic epitopes.

A protein of 58,000 Da molecular mass was purified from the supernatant fluid of a dialysis sac culture of *L. monocytogenes* serovar 4b by cation exchange chromatography. The purified protein, homogeneous on SDS-PAGE, was identified as listeriolysin O (LLO) and used to develop an indirect ELISA for the measurement of anti-LLO antibodies in sheep sera.

Oral dosing of lambs with *L. monocytogenes* serovar 4b daily for three days produced no clinical signs but conferred protection against bacteraemia following subsequent homologous subcutaneous challenges. Signs of systemic illness were unremarkable after the subcutaneous injections but between two and 61 days later neurological symptoms developed in six lambs. Histopathological lesions of listeric encephalomyelitis were demonstrated and it seems probable these cases developed as the result of infection ascending via the spinal nerves.

Antibodies to whole cell antigens were detected in the sera of challenged animals using serum agglutination tests and ELISA, and antibodies to LLO were detectable by immunoblotting and indirect ELISA. The subclass of antibody involved in the anti-LLO response was shown to be predominantly IgG. However, the competitive sandwich ELISA for the detection of anti-LTA antibodies was an unreliable indicator of infection. Seroconversion after oral dosing was apparently a consequence of host invasion since no antibody responses were detected when lambs were dosed with heat-killed *L. monocytogenes*. After oral dosing with viable *L. innocua* no antibody response to *L. monocytogenes* somatic antigens could be recognised.

In field studies the detection of anti-LLO antibodies by ELISA was shown to be useful for diagnosis of both septicaemic and abortion forms of listeriosis. However, anti-LLO antibodies were detected in clinically normal ewes from silage fed flocks suggesting that animals may be exposed to infection yet remain clinically normal as is consistent with the experimental studies. In confirmed cases of listeric encephalitis it was impracticable to diagnose the condition by the measurement of anti-LLO antibody titres or by the detection of LLO in CSF samples. Although unsuitable for the diagnosis of this form of infection the indirect ELISA and the measurement of anti-LLO antibodies may be useful in clarifying the pathogenesis of listeric encephalitis and the circumstances in which listeriosis occurs in sheep flocks.

In an examination of *Listeria* isolates from clinical cases of listeriosis nine isolates, all recovered from abortion cases, were identified as *L. ivanovii* and the remaining 108 as *L. monocytogenes*, with serovar 1/2a predominating. The majority of isolates were from cases of encephalitis and serovar 1/2b strains were confined to this form of disease.

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Introduction

Abbreviations and symbols used in text.

A	absorbance
APS	ammonium persulphate
B-cell	bone marrow derived lymphocyte
BHIB	brain heart infusion broth
BWB	blot wash buffer
c.f.u.	colony forming units
cm	centimetre
CSF	cerebrospinal fluid
DTE	dithioerythritol
DTH	delayed type hypersensitivity
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
gm	gram
g	acceleration of gravity
h	hour
HRP	horseradish peroxidase
HS	horse serum
HU	haemolytic unit
IgG	immunoglobulin G
IgM	immunoglobulin M
kDa	kilodalton
l	litre
LLO	listeriolysin O
LPS	lipopolysaccharide
LTA	lipoteichoic acid
M	molar concentration
mA	milliamp
mAb	monoclonal antibody
MES	2-[N-morpholino]ethanesulphonic acid
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar concentration
μg	microgram
μl	microlitre
NCTC	National Collection of Type Cultures
ng	nanogram
nm	nanometre
NT	not tested
O.D.	optical density
P	probability
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PE1	first phenol extract
PE2	second phenol extract
pH	pH-value
p.s.i.	pounds per square inch
RPMI	Rosewell Park Memorial Institute medium

SAT	serum agglutination test
sec	seconds
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
SLCC	Special *Listeria* Culture Collection
SLO	streptolysin O
T-cell	thymus derived lymphocyte
TEMED	N,N,N,N,tetramethylethylenediamine
TPB	tryptose phosphate broth
Tris	tris(hydroxymethyl)aminomethane
TSB	tryptic soy broth
V	volts
%	percentage
^{o}C	degrees Celsius
v/v	volume:volume
w/v	weight:volume

Chapter 1.

An introduction to *Listeria monocytogenes* and listeric infections, a review of the literature.

1.1 An historical appreciation

The bacterium now known as *Listeria monocytogenes* was first isolated from an epidemic disease of rabbits and guinea-pigs in a laboratory animal breeding unit (Murray *et al.*, 1926). The organism, a small, Gram positive rod, was tentatively ascribed to the genus *Bacterium* with the name *Bacterium monocytogenes*. The following year Pirie (1927) isolated a similar organism from a septicaemia of gerbils and named it *Listerella hepatolytica*. As a postscript to his own publication Pirie accepted the identity of the two organisms, the priority of the first report, and stated that a new genus *Listerella* should be created. The organism was thus termed *Listerella monocytogenes*. The generic name was later changed to *Listeria* as *Listerella* was a homonym already applied to a group of slime moulds (Pirie, 1940).

1.2 Taxonomy of the genus *Listeria*

The taxonomy of the genus *Listeria* is briefly described but a thorough discussion is beyond the scope of this introduction. The taxonomic position of the genus with regard to other genera is unclear and the reader is referred to the articles by: Stuart and Pease (1972); Stuart and Welshimer (1973); Jones (1975); Wilkinson and Jones (1977); Seeliger and Jones (1986) for further details.

1.2.1 Intrageneric relationship of the genus *Listeria*

Originally only a single species *Listeria monocytogenes* existed within the genus *Listeria* but avirulent, nonhaemolytic strains were found in environmental samples (Welshimer, 1968; Welshimer and Donker-Voet, 1971; Kampelmacher and van Noorle Jansen, 1972; Welshimer, 1975; Seeliger and Hohne, 1979) and the presence of haemolysis on blood agar is now recognised as an important determinant for the differentiation of *Listeria* species (Seeliger and Schoofs, 1979; Rocourt *et al.*, 1983). Classification of the genus remained unclear until an examination of the deoxyribonucleic acid (DNA) relatedness of sixty six strains formerly identified as *L. monocytogenes*. This revealed five distinct genomic groups with strong evidence that each represented a

distinct species (Rocourt *et al.*, 1982). Relatedness values were 18-58 per cent between groups, and differences between the thermal denaturation midpoints of the homoduplexes and heteroduplexes were more than 7.1°C.

Though the genomic groups can be distinguished on the basis of biochemical tests (Rocourt *et al.*, 1983), all are phenotypically similar: possessing peptidoglycan of the A1 gamma variation (Fiedler *et al.*, 1984), the same lipoteichoic acid structures (Ruhland and Fiedler, 1987), similar whole cell protein electrophoretic patterns (Lamont *et al.*, 1986), and with incomplete antigenic distinction (Seeliger and Hohne, 1979; Rocourt *et al.*, 1982) (Table 1.1).

However, the virulence of the proposed genomic groups differs widely (Rocourt *et al.*, 1982) and there are practical needs other than simply scientific reasons for subdividing the genus *Listeria*. To avoid misunderstanding and false interpretation of the role of *L. monocytogenes* in human and veterinary medicine the five genomic groups should be regarded as distinct species (Rocourt and Grimont, 1983). Seeliger and Jones (1986) adhere to this proposition and the species listed in the genus *Listeria* are: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri* and *L. seeligeri*. Three further species of *Listeria* are currently listed as

species *incertae sedis*. *L. murrayi* and *L. grayi* represent a single species (Stuart and Welshimer, 1974; Wilkinson and Jones, 1977) whose inclusion in the genus is controversial (Stuart and Welshimer, 1974; Rocourt *et al.*, 1987a). It differs from other *Listeria* in gluconate fermentation, lack of phosphoamidase, acid phosphatase (Rocourt and Catimel, 1985) and by being unsusceptible to *Listeria* bacteriophages (Rocourt *et al.*, 1985a). Only a single isolate of *L. denitrificans* exists and the natural habitat of the organism is unknown. The organism possesses a DNA base composition which differs considerably from other *Listeria* species (Collins *et al.*, 1983) and on the basis of 16S ribosomal ribonucleic acid cataloguing it has been proposed to transfer the organism to a new genus *Jonesia* (Rocourt *et al.*, 1987b).

Table 1.1.

Genomic groups of the genus *Listeria*

(according to Rocourt *et al.*, 1982)

Genomic group	Species name	Serovar
1	*L. monocytogenes*	1/2a, 1/2b, 1/2c
		3a, 3b, 3c
		4a, 4ab, 4b, 4c, 4d, 4e
		7
2	*L. ivanovii*	5
3	*L. innocua*	4ab
		6a, 6b, UD
4	*L. welshimeri*	6a, 6b
5	*L. seeligeri*	1/2b
		4c, 4d
		6b, UD

UD, undesignated serovars.

1.3 Identification of *Listeria*

1.3.1 Growth on artificial media

The original papers of Murray *et al.* (1926) and Pirie (1927) describe *L. monocytogenes* as a small, Gram positive rod, 1-2 μm in length and 0.5 μm wide. The organisms often lie parallel to each other in palisades, with a few thread like forms present in some cultures.

Descriptions of the organism (Murray *et al.*, 1926; Pirie, 1927; Gray and Killinger, 1966; Seeliger and Hohne, 1979) are in general agreement and full characteristics of *Listeria* species are given by Seeliger and Jones (1986). Growth occurs between 3 and 45^{o}C but the optimum range is 30 to 37^{o}C. The organism grows readily in aerobic or microaerophilic conditions but growth is absent or scant in a total absence of oxygen. Growth takes place at pH values as high as 9.6 but is inhibited by pH values lower than 5.6. Colonies are small, smooth, slightly flattened and milky white by reflected light. Gray and Killinger (1966) describe the colonies as having a characteristic blue/green colour when illuminated by obliquely transmitted light. Tumbling motility is best demonstrated by incubation of cultures at room temperature with none or sluggish motility being shown by organisms grown at 37^{o}C. Motile

organisms commonly possess between one and four subterminal flagellae.

1.3.2 Biochemical reactions of *Listeria*

Although relatively inactive biochemically *Listeria* strains produce catalase, are positive in the Voges-Proskauer reaction and hydrolyse aesculin. They are indole and oxidase negative, they do not hydrolyse urea or reduce nitrates and there is no liquefaction of gelatin. Interestingly Pirie (1927) described *L. monocytogenes* as nonhaemolytic. However as Gray and Killinger (1966) point out some strains show little haemolysis on blood agar plates.

All virulent strains of *Listeria* produce a haemolysin and the differentiation of species is partly based upon the presence or absence of haemolysis on blood agar (Groves and Welshimer, 1977). The haemolysis shown by *L. monocytogenes* can be enhanced by culture with *Staphylococcus aureus*, the so-called CAMP phenomenon (Brzin and Seeliger, 1975). Skala *et al.* (1982) state that the haemolysis of both *L. monocytogenes* and *L. ivanovii* can be enhanced by culture with *Rhodococcus equi*. Rocourt *et al.* (1983) claim only that of *L. ivanovii* strains is enhanced.

There are conflicting reports of the organism's fermentation reactions but the basal media, concentrations of carbohydrates, and pH indicators varied considerably. Gray and Killinger (1966) reported that under standard conditions hundreds of strains all produced acid but no gas from glucose, laevulose, salicin, and trehalose. In a study of sixty six strains (Rocourt *et al.*, 1983) the species of *Listeria* could be distinguished on the basis of a few simple tests (Table 1.2) and a further examination of seventy strains revealed no other distinguishing markers (Rocourt and Catimel, 1985).

Table 1.2.

Biochemical identification of the genus *Listeria*

(according to Rocourt *et al.*, 1983)

	Haemolysis of horse blood	CAMP test *Staphylococcus aureus*	CAMP test *Rhodococcus equi*	Production of acid from L-rhamnose	Production of acid from D-xylose	Production of acid from alpha-methyl D-mannoside
Listeria						
monocytogenes	+	+	-	+	-	+
ivanovii	+++	-	+	-	+	-
innocua	-	-	-	v	-	+
welshimeri	-	-	-	v	+	+
seeligeri	v	+	-	-	+	v

+ positive, +++ pronounced haemolysis, - negative, v variable reaction.

1.4 The cell surface of *Listeria*

1.4.1 The cell envelope

The cell envelope of prokaryotic microorganisms is characterised by the presence of two distinct components: the cytoplasmic membrane which bounds the cytoplasm and a strong outer cell wall which maintains the shape of the cell and prevents osmotic rupture of the cytoplasmic membrane (Davis *et al.*, 1980). The cell wall of *L. monocytogenes* is typical of a Gram positive bacterium with a thick peptidoglycan layer as the principal component (North, 1963; Fiedler and Ruhland, 1987). The peptidoglycan is of the A1 gamma variation described by Schleifer and Kandler (1972). Its structure consists of beta 1-4 linked N-acetyl glucosaminyl and N-acetyl muramyl residues cross linked by tetrapeptides through meso-diaminopimelic acid.

1.4.2 The teichoic acids

Teichoic acids are phosphate containing polymers of ribitol or glycerol covalently linked to the peptidoglycan (Archibald and Baddiley, 1966; Knox and Wicken, 1973) and are important components of Gram positive cell walls. Due to their anionic charge their function is thought to be the transport of ions across

the cell wall. Serologically reactive they feature in the classification of a number of bacterial genera including: *Lactobacilli, Staphylococci* and *Micrococci* (Knox and Wicken, 1973).

Using alkali extraction Kamisango *et al.* (1983) purified a serologically active teichoic acid from *L. monocytogenes* serovar 1/2a. This was a ribitol teichoic acid having a molecular mass of 12 kDa with glucosamine and rhamnose but no D-alanine ester substituents. Further biochemical studies within the genus *Listeria* revealed strain variation in the elucidated cell wall teichoic acid structures (Fiedler *et al.*, 1984; Fujii *et al.*, 1985; Uchikawa *et al.*, 1986a) and in serovars 4a and 4d novel repeating unit linkages through the glycosyl residues (Fujii *et al.*, 1985). A unique linkage between ribitol teichoic acid and peptidoglycan has been described in *L. monocytogenes* serovar 1/2a (Kaya *et al.*, 1985).

However, studies differ in their descriptions of the teichoic acid structures of *Listeria*. Fiedler *et al.* (1984) found a rhamnose dimer as the glycosyl substituent for serovar 1/2a whereas Kamisango *et al.* (1983) found only rhamnose monomer. Unlike Fiedler *et al.* (1984), Fujii *et al.* (1985) described no galactose in the teichoic acid of serovar 4a strains, no glucose or

galactose in a serotype 6 strain and glucose not galactose in a serovar 4e strain. Uchikawa *et al.* (1986a) found the ribitol teichoic acid from serovar 4a identical to that of serotype 6 strains. These discrepancies in structure may be the result of differences in growth media as relatively small changes in growth conditions can result in considerable variation in glycosyl substitution and even an absence of teichoic acids (Knox and Wicken, 1973). Alternatively the inconsistencies may result from the use of different bacterial strains or extraction methods.

1.4.3 Lipoteichoic acids

Lipoteichoic acids are classically glycerophosphate polymers covalently linked to glycolipid moieties of the cytoplasmic membrane. Consistently found in Gram positive bacteria these amphipathic molecules form micelles in solution. Though bound to the cytoplasmic membrane they are recognised as surface components being serologically important in *Lactobacillus fermenti* (van Driel *et al.*, 1973) and as group antigens for Group D and N *Streptococci* (Wicken and Knox, 1975).

The phenol extracted lipoteichoic acids of all *L. monocytogenes* strains have a similar structure (Hether and Jackson, 1983; Uchikawa *et al.*, 1986b; Ruhland and

Fiedler, 1987) with ester linked D-alanine and alpha linked galactose as substituents upon the glycerophosphate units of the hydrophilic chain. The chains are covalently linked either to galactosyl, glucosyl diacyl glycerol or phosphatidyl galactosyl, glucosyl diacyl glycerol.

Phenol/water extracts of *L. monocytogenes* are biologically active and reportedly contain the recognised markers for lipopolysaccharide (LPS): heptose and 2-keto-3-deoxyoctanate (Siddique and Srivastava, 1973; Conklin and Siddique, 1976; Wexler and Oppenheim, 1979; Singh *et al.*, 1981). The isolation of LPS from the unquestionably Gram positive *L. monocytogenes* is remarkable and others have been unable to confirm these findings (Hether and Jackson, 1983; Hofman *et al.*, 1985; Uchikawa *et al.*, 1986b; Fiedler and Ruhland, 1987; Ruhland and Fiedler, 1987). Uchikawa *et al.* (1986b) indicated that the earlier results may be ascribed to the isolation of lipoteichoic acid which possesses weak biological properties (Wicken and Knox, 1975; Hofman *et al.*, 1985).

1.4.4 Flagellae

Flagellae are whip like appendages responsible for motility of the organism. The main part of the flagellum consists of identical subunits of the protein flagellin

attached by a hook to a complex basal body in which rotational forces are created (Davis *et al.*, 1980). In *Listeria* the production of flagellin subunits is greatest at 20°C. *Listeria monocytogenes* serovar 4b flagellin subunits have a molecular mass in the region of 30 kDa (Peel, 1987) though a slight physical heterogeneity exists in the flagellins of six *L. monocytogenes* and *L. ivanovii* serovars (Peel, 1987).

A number of monoclonal antibodies recognise *Listeria* flagellar antigens (Farber and Spiers, 1987a; Peel, 1987). The monoclonal antibody of Peel (1987) was not fully characterised but the results were similar to those of Farber and Spiers (1987a) in which the monoclonal antibodies recognizing the C flagellar antigen of *L. monocytogenes* did not react with *L. ivanovii*, thus implying an unidentified difference in the C flagellar antigen of these species.

1.4.5 Examinations for the presence of a capsule

Using ferritin labelled antibody Smith and Metzger (1962) revealed a capsule on *L. monocytogenes* cells. However other workers have been unable to confirm this finding (Seeliger and Bockemuhl, 1968) and no capsule was recognised in electron microscope studies of the organism (North, 1963).

1.5 The immunochemistry of *Listeria*

Agglutination and absorption experiments with hyperimmune serum formed the basis of early serological studies on *Listeria* (Seastone, 1935; Webb and Barber, 1937; Julianelle, 1941) with the most important contribution made by Paterson (1939; 1940b). Using methods developed for *Salmonella* by Kaufmann and White he demonstrated somatic "O" and flagellar "H" antigens and described four serological types of *L. monocytogenes*. The examination of further strains extended this basic scheme to include more antigenic variants (Seeliger, 1961; Donker-Voet, 1966; Seeliger and Hohne, 1979) and the currently accepted Seeliger/Donker-Voet scheme is presented in Table 1.3. On the basis of somatic and flagellar antigens sixteen serovars of the genus *Listeria* are recognised, excluding the serologically distinct species *L. murrayi*, *L. grayi*, and *L. denitrificans*.

Table 1.3.

Serovars of the genus *Listeria*, (according to Seeliger and Jones, 1986)

Designation Paterson	Seeliger Donker-Voet	O antigens														H antigens				
1	1/2a	I	II	(III)[a]												A	B			
	1/2b	I	II	(III)												A	B	C		
2	1/2c	I	II	(III)													B		D	
3	3a		II	(III)	IV											A	B			
	3b		II	(III)	IV									(XII	XIII)	A	B	C		
	3c		II	(III)	IV									(XII	XIII)		B		D	
4	4a			(III)		(V)		VII			IX					A	B	C		
	4ab			(III)		V	VI	VII			IX	X				A	B	C		
	4b			(III)		V	VI									A	B	C		
	4c			(III)		V		VII								A	B	C		
	4d			(III)		(V)	VI			VIII						A	B	C		
	4e			(III)		V	VI			(VIII	IX)					A	B	C		
	5			(III)		(V)	VI			(VIII)		X				A	B	C		
	7			(III)										XII	XIII	A	B	C		
	6a(4f)			(III)		V	(VI | VII)			(IX)					XV	A	B	C		
	6b(4g)			(III)		(V	VI	VII)			IX	X	XI				A	B	C		

( )[a], not always present.

Osebold *et al.* (1965) were of the opinion that because of their thermostability and resistance to trypsin digestion the somatic antigen determinants of *L. monocytogenes* were polysaccharides. Hexose sugars form 20 per cent of the dry cell wall (Keeler and Gray, 1960) and Ullmann and Cameron (1969) extracted immunologically active carbohydrates from five serovars of *L. monocytogenes*. Precipitation by absorbed antisera was inhibited with simple sugars and the major antigenic determinant for serotypes 1 and 2 was shown to be rhamnose, for serovar 4a galactose and 4b glucose. The immunodeterminant for serotype 3 was not identified.

A correlation between serological properties and teichoic acid composition and structure has been suggested (Fiedler *et al.*, 1984; Fujii *et al.*, 1985) as a teichoic acid extracted from the cell walls of *L. monocytogenes* serovar 1/2a was serologically active and its precipitation by hyperimmune serum could be inhibited with rhamnose (Kamisango *et al.*, 1983). However, the discrepancies in the teichoic acid substituents (*vide supra*) and the absence of the reported somatic antigen determinant sugar galactose from serovar 4a strains (Fujii *et al.*, 1985; Uchikawa *et al.*, 1986a) suggests listeric somatic antigens cannot be entirely accounted for by cell wall teichoic acids (Uchikawa *et al.*, 1986a).

The similar structure of lipoteichoic acid in all strains of *L. monocytogenes* led Uchikawa *et al.* (1986b) and Ruhland and Fiedler (1987) to conclude that lipoteichoic acids were irrelevant to the serotyping of the genus *Listeria*. The amphipathic nature of the molecule and its ability to spontaneously attach to the membranes of erythrocytes has been utilised in serological tests and antibodies directed at listeric lipoteichoic acid can be detected in passive haemagglutination and inhibition assays. However, cross-reactions between lipoteichoic acids of *L. monocytogenes* and *Lactobacillus fermenti* have been described (Antonissen *et al.*, 1981) and lipoteichoic acid of *L. monocytogenes* binds antibody raised against *Streptococcus pyogenes* (Hether and Jackson, 1983).

1.6 Virulence factors of *Listeria*

1.6.1 Haemolysins

Mechanisms of virulence for *L. monocytogenes* are poorly understood and the nature, structure and biological functions of virulence determinants have only recently begun to be identified. Since all strains of *L. monocytogenes* isolated from clinical material are haemolytic on blood agar (Groves and Welshimer, 1977; Rocourt *et al.*, 1982) the haemolysin has been extensively studied as a potential virulence factor (Girard *et al.*,

1963; Njoku-Obi *et al.*, 1963; Jenkins *et al.*, 1964; Kingdon and Sword, 1970a; 1970b; Jenkins and Watson, 1971; Watson and Lavizzo, 1973; Lemeland *et al.*, 1974; Siddique *et al.*, 1974; Parrisius *et al.*, 1986; Gaillard *et al.*, 1986; Kathariou *et al.*, 1987; Geoffroy *et al.*, 1987; Berche *et al.*, 1987a; 1987b). The haemolysin is an antigenic, heat-labile, trypsin inactivated protein (Girard *et al.*, 1963; Njoku-Obi *et al.*, 1963). A similarity to sulphydryl-activated toxins, as typified by streptolysin O, is indicated by a decrease in its haemolytic activity with oxidation, an increase with reducing agents, and antigenic cross-reaction with antibodies to streptolysin O (Girard *et al.*, 1963; Njoku-Obi *et al.*, 1963; Jenkins *et al.*, 1964; Lemeland *et al.*, 1974). Its potential as a virulence factor is shown by its toxicity for polymorph neutrophils, ability to cause lysis of lysosomes, toxicity for continuous cell lines, and by the increase in clearance times of carbon from the blood of haemolysin treated mice (Njoku-Obi *et al.*, 1963; Kingdon and Sword, 1970a; 1970b; Watson and Lavizzo, 1973; Lemeland *et al.*, 1974; Farber and Spiers, 1987b).

The importance of the haemolysin as a virulence factor has been confirmed in the elegant work of Gaillard *et al.* (1986). Using transposon Tn1545 a nonhaemolytic mutant was derived from a haemolytic strain of *L. monocytogenes* serovar 1/2a. The nonhaemolytic mutant was unable to

replicate in host tissues and was avirulent for mice by intravenous challenge. Virulence and haemolytic activity were restored upon the spontaneous loss of the transposon. Identical results were reported using transposon Tn916 and the extracellular culture fluid of the nonhaemolytic mutant was shown to lack a 58 kDa protein (Kathariou *et al.*, 1987).

Purified by thiopropryl-activated affinity chromatography the haemolysin is a sulphydryl-activated toxin, termed listeriolysin O (LLO), with a molecular mass of 60 kDa (Geoffroy *et al.*, 1987). In common with other sulphydryl-activated toxins produced by a number of Gram positive bacteria (Table 1.4) LLO binds to cholesterol and has the ability to cause disruption of eukaryotic cell membranes (Alouf and Geoffroy, 1984). Uncharacteristically the activity of LLO is greatest between pH 5.5 and 6.0 which may be related to its intracellular function. Electron microscope studies (Armstrong and Sword, 1966) have shown the bacterium can escape the macrophage phagosome and multiply

Table 1.4.

Bacterial species producing sulphydryl-activated toxins (according to Alouf and Geoffroy, 1984)

Genus	Species	Toxin
Streptococcus	*S. pyogenes*	Streptolysin O
	S. pneumoniae	Pneumolysin O
Bacillus	*B. cereus*	Cereolysin
	B. thuringiensis	Thuringolysin O
	B. alvei	Alveolysin
	B. laterosporus	Laterosporolysin
Clostridium	*Cl. sordelli*	Bifermentolysin
	Cl. botulinum	Botulinolysin
	Cl. histolyticum	Histolyticolysin
	Cl. novyi type A	Oedematolysin O
	Cl. perfringens	Perfringolysin O
	Cl. septicum	Septicolysin O
	Cl. tetani	Tetanolysin
	Cl. chauvoei	Chauveolysin
Listeria	*L. monocytogenes*	Listeriolysin O

in the cell cytoplasm. The release of the organism is thought to be brought about by LLO causing disruption of phagosomal membranes (Geoffroy *et al.*, 1987). Berche *et al.* (1987a) and Gaillard *et al.* (1987) have confirmed the importance of LLO for intracellular survival and its recognition as a T-cell antigen has also been demonstrated (Berche *et al.*, 1987b).

Oddly LLO was not detected by western blotting in 26 out of 28 clinical isolates of *L. monocytogenes* (Parrisius *et al.*, 1986). However, there is strong historical evidence for a sulphydryl-activated haemolysin in many *L. monocytogenes* strains (Girard *et al,* 1963; Njoku-Obi *et al.*, 1963; Jenkins *et al.*, 1964; Lemeland *et al.*, 1974) and the failure to detect LLO may be attributed to a failure in purification of the toxin or to insensitivity of the assay.

Other haemolysins were detected in bacterial recombinants of *L. monocytogenes* harbouring 1 and 15 kbase inserts of chromosomal DNA (Leimeister-Wachter *et al.*, 1987). The production of these recombinant proteins by native strains has not been described and their significance is unclear. Phospholipase activity is associated with *L. monocytogenes* (Fuzi and Pillis, 1962; Jenkins and Watson, 1971; Siddique *et al.*, 1974). However, evidence from the insertion of a single transposon in the structural gene of LLO with loss of both haemolytic activity and virulence (Gaillard *et al.*,

1986; Mengaud *et al.*, 1987) argues against the importance of other toxins.

1.6.2 Catalase and superoxide dismutase activity

Neither superoxide dismutase or catalase activity contribute to the virulence of *L. monocytogenes*. Two catalase negative mutants have been reported which retained their virulence *in vivo* (Gaillard *et al.*, 1986) and no statistically significant correlation between virulence and catalase or superoxide dismutase activities of five strains of *L. monocytogenes* could be shown (Welch *et al.*, 1979; Welch, 1987). Catalase and superoxide dismutase may be unnecessary for virulence since the organism avoids the oxidative attack of the cell by escaping from the phagosome and replicating within the macrophage cytoplasm.

1.6.3 Cell wall associated virulence factors

Crude cell wall of *L. monocytogenes* has many biological effects, being a B-cell mitogen (Petit and Unanue, 1974; Cohen *et al.*, 1975; Saiki *et al.*, 1982; Hether *et al.*, 1983; Paquet *et al.*, 1986) and a T-cell independent adjuvant (Campbell *et al.*, 1976; Paquet *et al.*, 1986). It is also capable of increasing nonspecific resistance to infection (Petit and Unanue, 1974; Rodriguez *et al.*, 1974; Paquet *et al.*, 1986), inducing *in vitro* and *in vivo*

cytocidal activity of macrophages (Saiki *et al.*, 1982; Hether *et al.*, 1983; Paquet *et al.*, 1986) and increasing natural killer cell activity (Paquet *et al.*, 1986). Peptidoglycan extracted from the cell wall may be responsible for many of these immunological properties (Saiki *et al.*, 1982; Paquet *et al.*, 1986) though Hether *et al.* (1983) indicated that with increasing purification and loss of lipids there is a concomitant loss of mitogenic and adjuvant properties, and Hofman *et al.* (1985) found a phenol/water extract of *L. monocytogenes* possessed both mitogenic and adjuvant effects. An ether extract of *L. monocytogenes* containing proteins, polysaccharides and lipids resulted in a decrease in the LD_{50} when injected into mice with *L. monocytogenes* (Mara *et al.*, 1974b). A nonspecific decrease in resistance to infection is also induced by protease treated cell walls (Baker and Campbell, 1978). However, these extracted cell wall products and studies of their effects are unrepresentative of the natural disease. The role of their varied and at times conflicting immunological effects on the pathogenesis of infection remains unclear.

1.6.4 Monocytosis producing agent

A monocytosis producing agent (MPA) has been extracted from *L. monocytogenes* cell walls (Stanley, 1949). With a molecular mass of under 1,000 and reportedly containing no

amino acids or carbohydrates its effects are mediated via an endogenous factor (Galsworthy, 1987). The failure of extracts from *L. innocua* to stimulate a monocytosis suggests MPA may have a role in virulence.

1.6.5 Cell wall defective variants

The difficulties in demonstrating or culturing *L. monocytogenes* from some clinical cases has lead to the suggestion that cell wall defective variants may be important in the pathogenesis of listeriosis (Gray and Killinger, 1966). However, the derivation of typical organisms from L-forms isolated from a human case of listeric encephalitis (Charache, 1970) fails to prove that the variants were involved in the disease process. Rather they may have been induced by antibiotic treatment as penicillin can induce stable cell wall defective variants of *L. monocytogenes* (Louria *et al.*, 1967; Brem and Eveland, 1968; Edman *et al.*, 1968). A stable L-form when injected into mice was avirulent and did not revert (Louria *et al.*, 1967).

1.6.6 The *in vivo* environment

The recognition that the *in vivo* environment has considerable influence upon bacterial growth has been of immense importance for the elucidation of virulence determinants. For example, *Bacillus anthracis* recovered from the thoracic and peritoneal cavities of infected animals produces a lethal toxin and physically differs from *in vitro* grown organisms (Smith *et al.*, 1953a; 1953b; 1955).

Within the host a major constraint upon bacterial growth is the low availability of iron because of its binding to glycoproteins such as transferrin or incorporation within molecules such as ferritin and haem (Griffiths, 1983). Under iron restricted conditions a number of Gram negative bacteria including: *Salmonella typhimurium, Escherichia coli, Klebsiella pneumoniae, Pasteurella haemolytica* and *Pseudomonas aeruginosa*, produce iron regulated outer membrane proteins which in some cases are receptors for high affinity iron binding molecules or siderophores (Griffiths, 1983; Donachie and Gilmour, 1988). Though the iron acquisition mechanisms in Gram positive bacteria remain largely obscure *Staphylococcus epidermidis* is capable of obtaining iron from transferrin (Williams *et al.*, 1988). Following *in vitro* growth of *L. monocytogenes* in a low iron medium no specific acquisition mechanism

could be identified though a low molecular mass reductant was produced which rapidly reduced iron from transferrin (Cowart and Foster, 1985). The injection of mice with iron increases the *in vivo* growth rate of *L. monocytogenes* and reduces the bacterial LD_{50} (Sword, 1966).

A further influence of *in vivo* conditions may be the presence of preferred nutrients in certain tissues. Erythritol is a preferred nutrient for *Brucella abortus* and analogues of erythritol inhibit the organism's growth both *in vivo* and *in vitro* (Smith *et al.*, 1965). Further, the organism's ability to cause abortion is restricted to cattle, sheep, goats and pigs; species in which erythritol is present in the placentae (Smith *et al.*, 1962). It is particularly intriguing that *L. ivanovii* has a restricted pathogenicity, being a cause of abortion solely in sheep (Ivanov, 1962; MacLeod *et al.*, 1974; Seeliger *et al.*, 1984), and an examination of the possible tropisms of *Listeria* species merits investigation.

Currently the influence of *in vivo* conditions on the production of virulence determinants of *L. monocytogenes* has not been extensively studied. Though the organism survived for prolonged periods in diffusion chambers implanted in the peritoneal cavities of mice (Osebold *et al.*, 1970a; 1970b) only the colonial morphology of the surviving organisms was examined (Osebold *et al.*, 1970b).

1.6.7 Invasion of the host

Multiplication within the host represents only one part of the infectious process and an additional aspect to the virulence of *L. monocytogenes* is its ability to penetrate and invade host tissues. Cell penetration has been shown to be a characteristic of pathogenic species and the CaCo-2 enterocyte-like cell line can be invaded *in vitro* by *L. monocytogenes* and *L. ivanovii* but not by the nonpathogenic species *L. innocua, L. welshimeri* or *L. seeligeri* (Gaillard *et al.*, 1987). Though listeriolysin O is essential for intracellular multiplication it is unnecessary for invasion since a nonhaemolytic mutant is as capable of entering these cells as the haemolytic revertant strain (Gaillard *et al.*, 1987). The characteristics which mediate bacterial attachment and penetration of cells have yet to be determined.

1.7 Distribution of *Listeria*

Listeria species including *L. monocytogenes* are widespread having been isolated from surface soils (Welshimer and Donker-Voet, 1971; Weis, 1975a), decaying vegetation and pasture herbage (Welshimer, 1968; Welshimer and Donker-Voet, 1971; Weis, 1975a), silage (Gray, 1960), sewage sludge (Watkins and Sleath, 1981; Fenlon, 1985), factory effluents and river waters (Watkins and Sleath, 1981). The

faeces of healthy animals often contain *L. monocytogenes* (Gray and Killinger, 1966; Kampelmacher and van Noorle Jansen, 1969; Gronstol, 1979a) and Rocourt and Seeliger (1985) state that the natural habitat for all *Listeria* species is the environment.

In a study of the distribution of 573 strains, *L. monocytogenes* was found to have a global distribution and *L. ivanovii* was identified in Australasia, Europe and USA. Though isolates from pathological material were invariably shown to be *L. monocytogenes* or *L. ivanovii*, all species of *Listeria* were isolated from the gastrointestinal tract of healthy animals (Rocourt and Seeliger, 1985). Gronstol (1980a) reports that *Listeria* species may be occasionally isolated from the tissues of normal animals.

1.8 Listeriosis in animals and man

Listeria monocytogenes is a cause of infectious disease in both animals and man. Therefore in addition to the description of the organism this introduction attempts to provide an overview of the clinical forms of disease, to discuss the pathogenesis and epidemiology of infection, mechanisms of host resistance and current diagnostic methods.

1.8.1 Listeriosis in animals

Listeriosis in animals has been extensively reviewed by: Murray (1955); Seeliger (1961); Gray and Killinger (1966). The disease is truly widespread having been recorded in more than forty species of wild and domesticated animals and in countries over six continents (Seeliger, 1961).

Listeriosis is of major importance in the domesticated ruminants: cattle, sheep and goats but in the UK is most common in sheep (Anon., 1988a). A number of conditions are associated with infection; encephalitis and uterine infections being most frequently identified (Wilesmith and Gitter, 1986). Remarkably it is the exception for different listeric conditions to occur in the same flock or herd (Ladds *et al.*, 1974; Sullivan, 1985) and Wilesmith and Gitter (1986) record overlap of different clinical manifestations in only seven of 75 affected flocks.

1.8.1.1 Encephalitis

Gill (1931) is credited with the first description of listeric encephalitis. He described a widespread neurological disease of sheep in New Zealand known locally as "circling disease." No gross pathological lesions were apparent but histological examination of the brain revealed purulent foci and perivascular cuffs in the corpora

quadrigemina, pons and medulla. *Listeria monocytogenes* was readily isolated from the lesions (Gill, 1933) and listeric encephalitis of sheep, cattle and goats has since been described throughout the world (Jungherr, 1937; Graham, 1939; Biester and Schwarte, 1939; Pillai, 1962; Gitter *et al.*, 1965; Vandegraff *et al.*, 1981; Istvan, 1982).

In sheep, cattle and goats clinical signs of listeric encephalitis are a consequence of the lesions in the brain stem (Rebhun and deLahunta, 1982). Individual cases vary but common symptoms are: dullness, turning or twisting of the head to one side, and walking in circles. Unilateral facial nerve paralysis causes drooping of the eyelid and ear and occasionally because of partial pharyngeal paralysis the animal drools saliva. In sheep and goats recumbency and death occur within two or three days but in cattle the duration of illness is longer. Depending on the stage of infection the rectal temperature may be normal or elevated.

Gross pathological lesions are rarely observed. The pathognomonic histological lesions are usually unilateral and consistently most severe in the medulla oblongata and pons. Lesions are less frequent in the cerebellum, cervical spinal cord and diencephalon and when present in these sites are of lesser severity. The characteristic lesion is a focus of inflammatory cells with adjacent

perivascular cuffs. In severe cases lesions may coalesce to affect large areas of brain tissue. Meningitis is often present developing secondary to the parenchymal lesions. The ependyma and choroid plexi are rarely affected (Cordy and Osebold, 1959; Ladds *et al.*, 1974; Charlton and Garcia, 1977; Sullivan, 1985). Intriguingly in encephalitis cases *L. monocytogenes* is invariably isolated only from the brain (Urbaneck, 1962a; Charlton and Garcia, 1977).

Asahi *et al.* (1957) described mononuclear neuritis throughout the length of one or both trigeminal nerves in spontaneous cases of listeric encephalitis in five goats and two sheep. Lesions in other cranial nerves were confined to within a short distance of the brain. Similarly, Urbaneck (1962a) reported endoneuritis and perineuritis of the trigeminal nerves from 23 field cases. Lesions were often present only in one of the lesser branches and frequently in discrete sections of single nerve bundles. The presence, distribution and extent of lesions in the cranial nerves was confirmed in further studies (Groch, 1976; Charlton and Garcia, 1977) but contrary to the findings of Urbaneck (1962a) endoneuritis not perineuritis was regarded as the major lesion (Charlton and Garcia, 1977).

In seventeen natural ovine cases the inflammatory foci in the brain parenchyma consisted of macrophages, neutrophils or both, with neutrophils rarely the dominant cell type (Charlton and Garcia, 1977). In other studies neutrophils have been described as being dominant (Olafson, 1940; Cordy and Osebold, 1959). Urbaneck (1962a) found the cell type to vary not only between individuals but between sections from the same brain. The perivascular cuffs consist predominantly of lymphocytes with histiocytes, plasma cells and occasional neutrophils. Though numbers differed from case to case bacteria with the morphological appearance of *Listeria* have been described within the parenchymal lesions. These are often detectable in phagocytes and scattered neurones and axons (Charlton and Garcia, 1977; Charlton, 1977) but not in the perivascular cuffs (Olafson, 1940; Cordy and Osebold, 1959; Barlow, 1983).

1.8.1.2 Uterine infections

Listeric abortion caused by *L. monocytogenes* is a well recognised entity occurring in many species of domesticated animal (Paterson, 1940a; Stockton *et al.*, 1954; Njoku and Dennis, 1973; Ladds *et al.*, 1974; Jubb *et al.*, 1985). The recorded incidence in UK is highest in sheep (Anon., 1988a) and though cases are usually sporadic (Jubb *et al.*, 1985) in exceptional circumstances 50 percent of the flock have been affected (Low and Renton, 1985). Reports of abortion

are usually confined to the last trimester possibly because abortions are more obvious at this time. Placental lesions are: pin-point, yellowish, necrotic foci involving the tips of the cotyledonary villi with a focal to diffuse intercotyledonary placentitis covered in a red/brown exudate. The fetus is usually autolytic and pleural and peritoneal effusions are common with miliary necrotic foci often scattered throughout the liver and spleen. Histologically these foci show coagulative necrosis of hepatocytes and infiltration to variable degrees by macrophages and neutrophils (McDonald, 1967; MacLeod *et al.*, 1974; Barlow, 1983; Jubb *et al.*, 1985). Both *L. monocytogenes* and *L. ivanovii* are recorded as causing abortion in sheep (Paterson, 1940a; MacLeod *et al.*, 1974; Rocourt and Seeliger, 1985) and in abortion specimens can be readily isolated from fetal and placental lesions and the fetal stomach contents.

1.8.1.3 Septicaemia

Septicaemia is relatively uncommon and generally, though not invariably, occurs in the neonate possibly as an extension of intrauterine infection. The most consistent lesion is focal hepatic necrosis with pin-point greyish, white nodules throughout the liver. Lesions are also present in the spleen but rarely in other tissues. The histological appearance is of multiple, focal areas of

necrosis with invasion by polymorphs and mononuclear cells. The organism can be readily demonstrated in the lesions and is easily isolated on direct culture (Seeliger, 1961; Gray and Killinger, 1966).

1.8.1.4 Other conditions

Iritis and keratoconjunctivitis caused by *L. monocytogenes* have occasionally been recorded in both cattle and sheep (Kummeneje and Mikkelsen, 1975; Morgan, 1977). The condition is often unilateral and usually occurs during winter in silage fed animals. Bovine mastitis has been reported but with few documented cases (Gitter *et al.*, 1980). Clinically the condition presents as abnormal milk secretion and swelling of the affected quarter. In all the cases reviewed the response to antibiotic treatment was poor and the organism was excreted for prolonged periods.

In monogastric animals listeriosis is uncommon though septicaemia and meningoencephalitis have been recorded (Gray and Killinger, 1966). Infection in birds causes a septicaemia and myocardial necrosis (Seastone, 1935). Infection of rabbits with sublethal doses characteristically induces a marked monocytosis (Murray *et al.*, 1926; Osebold and Inouye, 1954a; Gray and Killinger, 1966) and in laboratory animals and rodents a septicaemia as originally described by Murray *et al.* (1926). At

postmortem examination lesions are similar to those seen in other species and isolation of the organism is relatively easy.

1.8.2 Pathogenesis of listeriosis in animals

1.8.2.1 Pathogenesis of encephalitis

For many years the pathway by which *L. monocytogenes* reaches the brain and causes encephalitis has been the subject of research and speculation. Gill (1931) was of the opinion that *L. monocytogenes* invaded the brain via the nasal passages and lymphatics but provided no explanation for the absence of olfactory lobe lesions. Graham *et al.* (1940a) failed to produce listeric encephalitis by intravenous injection, conjunctival instillation and intraruminal inoculation. Osebold and Inouye (1954b) reported that intracarotid, intracerebral, intravenous, subcutaneous, intramuscular and intradermal injections all failed to produce typical listeric encephalitis. Similarly Asahi *et al.* (1957) were unsuccessful with intranasal, subcutaneous, intraperitoneal, intravenous, intracarotid and intracerebral challenges. In contrast Osebold and Sawyer (1956) claimed intracarotid injection produced encephalitis typical of natural cases, however *L. monocytogenes* was present in the visceral organs and the lesion described included a marked choroiditis. Generally

intracarotid and intracerebral inoculation produces a diffuse encephalitis (Gill, 1933; Jungherr, 1937; Biester and Schwarte, 1939; Graham *et al.*, 1940a; Olson *et al.*, 1953). Olafson (1940) points out the pattern of the histological lesions and clinical symptoms following intracerebral and intracarotid injection are different to those of the natural disease. He believed infection arose following a rhinitis but did not suggest how the organism reached the brain. Osebold and Inouye (1954a) instilled into rabbits' noses approximately $1x10^{10}$ c.f.u. *L. monocytogenes*. This produced severe nasal discharges and at 72 hours neurological symptoms in four out of seven animals, examination revealed massive abscessation of the cribriform plate and extensive meningitis. In sheep the results of intranasal exposure were not analogous to natural encephalitis (Osebold and Inouye, 1954b).

Asahi *et al.* (1957) state that none of the above routes satisfactorily explains the unilateral nature of the pathological lesions, their topographic distribution and the absence of the organism in tissues other than the brain. These workers produced listeric encephalitis in mice by applying *L. monocytogenes* to minute puncture wounds on the lips and oral mucosae. The most successful route was inoculation of the lip with two patterns of disease reported. No neurological symptoms were apparent in the first four days after challenge, histological lesions were

of septicaemia and *L. monocytogenes* was cultured from all organs. After four days neurological symptoms and histological lesions were typical of listeric encephalitis and the organism was isolated primarily from the brain stem. Sequential studies revealed mononuclear cell infiltrations at the root of the trigeminal nerves within 24 hours and in the medulla oblongata by day four. Four of eleven goats exposed in this way developed neurological disease 17 to 28 days after challenge and histological lesions resembled those of the natural disease. A goat challenged by conjunctival instillation of the organism showed no symptoms but developed a trigeminal neuritis and in the brain stem mild glial and mononuclear cell infiltrations. The authors suggest the results of these experiments and others involving the feeding of *L. monocytogenes* mixed with coarse foodstuffs support the hypothesis, derived from field evidence (*vide supra*), that in listeric encephalitis the organism reaches the brain via the trigeminal nerves following invasion of the oral or nasal mucosae.

Cordy and Osebold (1959) attempted to confirm the findings of Asahi *et al.* (1957) and produced encephalitis in 25 out of 89 mice inoculated by a variety of routes. Animals with encephalitis died after a longer period (mean 9.3 days) than those dying of septicaemia (mean 4.9 days). However, oral scarification and inoculation produced no lesions in

the distal extremities of the trigeminal nerves. The absence of neuritis, the longer incubation period for encephalitis and the presence of mild encephalitis in five clinically normal mice was taken to imply that encephalitis resulted from haematogenous spread and occurred in mice resisting an overwhelming septicaemia. Lesions in the proximal parts of the trigeminal nerves were thought to be the result of infection descending from the brain stem. The authors suggest that in sheep haematogenous spread of the organism occurs and there is a vascular basis to the topographic distribution of lesions in the brain.

After the direct injection of *L. monocytogenes* into the facial or trigeminal nerves of sheep Borman *et al.* (1960) were unable to determine whether infection of the brain was via the cranial nerves or the result of haematogenous spread. Clinical signs of encephalitis, but including unusual signs such as lameness, developed in nine animals 18 to 37 days after injection. Two were shown to be bacteraemic immediately after challenge and histological examination of the cranial nerves gave inconsistent results. In four sheep lesions were found throughout the injected nerves and in two were localised at the sites of inoculation. However, in others no lesions were identified or lesions were present in uninjected nerves.

Other reports are in support of infection of the brain via the trigeminal nerves. After the injection of *L. monocytogenes* into the trigeminal nerve of ten sheep two developed clinical symptoms of neurological disease at ten and 14 days. Histological encephalitis was recorded in nine but with differences in respect of cell character and intensity of lesions (Urbaneck, 1962b). Histological lesions were confined to the pons and medulla, were mainly unilateral and most severe on the side of the injected nerves. The possibility of haematogenous spread is dismissed by the author as the direct association of parenchymal lesions with capillaries was rarely observed. Secondly the isolation of the organism from visceral organs he assumed to be the result of terminal spread from the brain. Schleicher *et al.* (1968) report a topographic relationship between site of injection, spread of neuritis and localisation of brain lesions. Barlow and McGorum (1985) used a novel experimental method and inoculated *L. monocytogenes* into the pulp cavities of premolar or incisor teeth. Subsequently, 17 of 21 sheep were found to have a trigeminal neuritis and perineuritis, 14 had histological lesions of encephalitis, and between days 20 and 41 six developed neurological symptoms typical of listeric encephalitis.

1.8.2.2 Pathogenesis of uterine infections

The gravid uterus is highly susceptible to infection and abortion is readily reproduced by the intravenous injection of pregnant ewes with *L. monocytogenes* or *L. ivanovii* (Paterson, 1940a; Molello and Jensen, 1964; Smith *et al.*, 1968; Njoku *et al.*, 1972). However, oral infection does not consistently produce abortion (Paterson, 1940a; Gray *et al.*, 1956; Gitter *et al.*, 1986b). The pathogenesis of fetal infection is thought to be haematogenous spread from the placenta with abortion a consequence of fetal death due to septicaemia and placental insufficiency (Molello and Jensen, 1964; Smith *et al.*, 1970; Njoku and Dennis, 1973).

After intravenous challenge abortion occurs in five to twelve days and lesions are said to be identical to those seen in natural infections (*vide supra*). The placenta is covered in a red/brown exudate, shows cotyledonary oedema, degeneration of the chorionic epithelium and a marked vasculitis (Molello and Jensen, 1964; Smith *et al.*, 1970; Njoku and Dennis, 1973). In the fetus there are areas of focal hepatic necrosis associated with blood vessels. In these lesions Smith *et al.* (1970) and Njoku and Dennis (1973) report no evidence of an inflammatory response. Ladds *et al.* (1974) suggest that in older fetuses infection is confined to the liver and placenta and involves an inflammatory response whilst infection at an earlier stage

is more generalised and involves no inflammation. Organisms are rarely cultured from maternal tissues but using Gram's stain are demonstrable in both fetus and placenta and are readily isolated from these sites on direct culture. (Paterson, 1940a; Smith *et al.*, 1970).

1.8.2.3 Pathogenesis of septicaemia

Given a sufficiently large inoculum septicaemia is readily produced by the intravenous injection of *L. monocytogenes* in a variety of experimental animals (Murray *et al.*, 1926; Gill, 1937; Jungherr, 1937; Osebold and Inouye, 1954a; Gray and Killinger, 1966). Minimal clinical responses occur after the subcutaneous injection of sheep or goats with *L. monocytogenes* (Gill, 1933; Olafson, 1940; Olson *et al.*, 1950; Osebold and Inouye, 1954b; Osebold and Sawyer, 1956) though a transient pyrexia and the production of antibodies to whole cell antigen have been recorded (Olson *et al.*, 1950; Osebold and Inouye, 1954b). These workers report that ruminants, unlike laboratory animals, develop a neutrophilia following challenge with sublethal doses.

Despite the evidence of the importance of the oral route in natural infections there are few detailed reports on the oral dosing of animals with *L. monocytogenes* and the majority of these are confined to laboratory animals. *In vivo* studies in mice have shown that after oral challenge

with high doses virulent organisms can invade host tissues and replicate in the spleen and liver (Audurier *et al.*, 1980; 1981). In contrast avirulent strains were unable to penetrate further than the mesenteric lymph nodes (Audurier *et al.*, 1981). Oral dosing with virulent strains also resulted in systemic infection in newborn piglets (Busch *et al.*, 1971).

Oral challenge of sheep with *L. monocytogenes* results in a transient pyrexia and clinical symptoms which are so slight as to be unremarkable (Gill, 1933; Osebold and Inouye, 1954b; Gray and Killinger, 1966). Osebold and Inouye (1954b) report on the presence of mononuclear cells and polymorphs in the lamina propria of the bowel and the isolation of *L. monocytogenes* from the tissues of a ewe seven days after oral dosing with approximately $2x10^{11}$ colony forming units.

The site of penetration after oral dosing is unclear. A gross enteritis was demonstrated nine hours after the oral challenge of starved guinea-pigs with 10^{10} c.f.u. of *L. monocytogenes*. Light and electron microscopy revealed bacterial invasion in the ileum predominantly in epithelial cells at the tips of villi with the organism both free in the cell cytoplasm and within membrane bound vacuoles. Replicating forms were common and multiplication eventually resulted in epithelial cell damage. Movement between cells

was observed and at 22 hours after infection organisms were mainly within polymorph leucocytes and macrophages in the villus stroma (Racz *et al.*, 1972). Thus in guinea-pigs at least an epithelial phase of infection is important and the organism is able to cross the defensive barrier of the gut by invasion and destruction of epithelial cells. In contrast, in SPF mice MacDonald and Carter (1980) were unable to isolate *L. monocytogenes* from the intestinal mucosa dissected free of Peyer's patches. However doses of more than 2.5×10^8 c.f.u. initiated infection and within 48 hours organisms had disseminated to the mesenteric lymph nodes, spleen and liver. Lower doses resulted in inconsistent infection of the Peyer's patches and no further spread. Zachar and Savage (1979) suggest that starvation may be important in allowing colonization of the gastrointestinal tract, as after oral dosing of SPF mice with 5×10^7 c.f.u. *L. monocytogenes* the organism was only isolated from the bowel of those animals starved for 48 hours beforehand.

1.8.3 Predisposing factors

Olson and Segre (1956) described a listeria enhancing agent which when given intranasally in combination with *L. monocytogenes* resulted in listeric encephalitis in six out of seven lambs 18 to 22 days later. The agent was not defined but was isolated from the blood of pyrexic lambs

and maintained by passage through eggs. It was sterile on bacterial culture and caused no cytopathic effect in tissue culture maintained cell lines.

Inclement winter weather may precipitate listeriosis (Gitter et al., 1965; Vandegraff *et al.*, 1981; Loken and Gronstol, 1982; Wardrope and MacLeod, 1983). However, this may be an indirect effect as animals are either forced to eat silage contaminated by *L. monocytogenes* (Loken and Gronstol, 1982; Wardrope and MacLeod, 1983) or are exposed to heavy environmental contamination by *Listeria* (Vandegraff *et al.*, 1981) possibly as a result of the congregation of animals. Low and Renton (1985) have described a severe listeriosis outbreak in which the ewes were protected from the weather by housing.

The role of intercurrent disease and immunosuppression is unclear. Vandegraff *et al.* (1981) describe a number of conditions associated with listeriosis such as hypocupraemia, anaemia and intestinal parasitism, but Wilesmith and Gitter (1986) found no evidence to support an interaction with intercurrent disease. In a series of experiments marked for their lack of success in producing clinical disease no evidence was produced that immunosuppressive drugs including: prednisolone, cyclophosphamide, niridazole (Gronstol, 1980c), infection by *Eperythrozoon ovis* (Gronstol and Overas, 1980a) or tick-

borne fever (Gronstol and Overas, 1980b) could predispose listeric infections.

1.8.4 Listeriosis in man

Listeric infections in man have been reviewed by Seeliger (1961); Gray and Killinger (1966); Neiman and Lorber (1980); Seeliger and Finger (1983); McLauchlin (1987). *Listeria monocytogenes* is the significant pathogen causing: meningitis, encephalitis, septicaemia, uterine infections, endocarditis and miscellaneous localised conditions. In the USA 33 per cent of 660 listeriosis cases were associated with pregnancy (Ciesielski *et al.*, 1988) and in the UK 248 of 722 cases between 1967 and 1985 (McLauchlin, 1987). Infection during pregnancy is associated with abortion, stillbirth or septicaemia of the newborn (granulomatosis infantiseptica). Healthy adults are rarely susceptible to serious infection other than when pregnant and listeriosis is most common in infants, the elderly, renal transplant patients, or patients suffering from underlying illness such as neoplasia, particularly lymphomas and leukaemias, alcoholism, or diabetes mellitus (Louria *et al.*, 1967; Neiman and Lorber, 1980; Pollock *et al.*, 1984; McLauchlin, 1987). In renal transplant patients the prevention of tissue rejection by the use of corticosteroid drugs and azathioprine is thought to render

the patients more susceptible to listeriosis (Gantz *et al.*, 1975; Niklasson *et al.*, 1978).

The incidence of listeriosis in industrialised nations varies from 10 to 50 cases per million population (Anon., 1988b). Typically these are sporadic cases in which the source of infection is unknown. Epidemic outbreaks have been described associated with the consumption of contaminated foodstuffs including pasteurised milk and soft cheeses (Schlech *et al.*, 1983; Fleming *et al.*, 1985; James *et al.*, 1985). The recognition of the widespread occurrence of *L. monocytogenes* in foodstuffs, particularly dairy and meat products (Anon., 1988b), has led to considerable public concern and the publication of guidelines for both food producers and consumers (Anon., 1988b). No marked seasonality, no preponderance of patients from rural environments and usually no direct links to animal infections have been found (Monnet, 1975; Pollock *et al.*, 1984; Ciesielski *et al.*, 1988; Anon., 1988b).

Meningitis is the most common form of the disease, with encephalitis a rare occurrence (Niklasson *et al.*, 1978; Katz *et al.*, 1979; Kennard *et al.*, 1979; Neiman and Lorber, 1980; Pollock *et al.*, 1984; Felten *et al.*, 1986). However, the similarity of these encephalitic cases to encephalitis in sheep has been noted (Kennard *et al.*, 1979; Pollock *et al.*, 1984). Pollock *et al.* (1984) suggested that the

development of encephalitis represents a modified response to infection as a result of partial immunity from previous exposure to *L. monocytogenes*.

1.9 Epidemiology of listeriosis in sheep

Recently the pattern of disease in the United Kingdom has changed from sporadic cases to flock outbreaks (Anon., 1983; Wilesmith and Gitter, 1986) and the incidence recorded by Veterinary Investigation Centres has increased from 34 incidents in 1977 to 337 in 1987 (Anon., 1988a). Though an increase in this period could be expected with the expansion in the UK sheep industry the diagnoses for erysipelas, another bacterial disease, have remained constant at 47 incidents in 1977 and 46 in 1987.

In a survey of sixty flocks Wilesmith and Gitter (1986) report a variable number of encephalitis cases with a mean attack rate of 2.5 per cent in adults and a range of 0.1 to 13.3 per cent. Similar incidences have been recorded elsewhere (Graham, 1939; Olafson, 1940; Vandegraff *et al.*, 1981). In the UK, listeriosis in sheep has a marked seasonality and though abortion is obviously associated with pregnancy, the majority of encephalitis cases occur in February and March (Anon., 1983; Anon., 1988a). The reason for this seasonal distribution is obscure but the peak of infection is coincidental with late pregnancy when there

are fundamental changes in the animals' immune status (*vide infra*). Though Barlow and McGorum (1985) claim that few cases occur between one and two years of age their data covers few individuals and generally no definite age incidence has been recognised (Olafson, 1940; Vandegraff *et al.*, 1981; Wardrope and Macleod, 1983; Wilesmith and Gitter, 1986).

1.9.1 Enteric carriage of *Listeria*

The faecal carriage rate of *Listeria* species by sheep has been examined on a number of occasions (Gronstol 1979a; 1979b; 1980b; Loken *et al.*, 1982). However the relevance of these studies is unclear as isolation depended upon prolonged cold enrichment, the bacteria were not enumerated and through a lack of appreciation of different *Listeria* species the isolates were not identified as pathogenic strains. It has been clearly shown that *L. monocytogenes* may be present in the faeces and intestinal contents of normal animals (Rocourt and Seeliger, 1985) however it is uncertain whether the organism is maintained for long periods in the bowel and whether passive faecal carriage is of epidemiological importance.

1.9.2 Relationship to silage feeding

Silage feeding has been associated with listeriosis for many years (Olafson, 1940; Gray, 1960) and Wilesmith and Gitter (1986) found that the incidence of listeriosis increased in flocks when silage feeding was introduced. On occasions silage involved in listeriosis outbreaks has been shown to contain more than 10^7 *L. monocytogenes* c.f.u. per kilogram (Gray, 1960; Low and Renton, 1985; Fenlon, 1986). The seasonal occurrence of listeriosis together with the increase in both severity and numbers of outbreaks may be explained by the increasing popularity and extensive use of silage as a feedstuff. However whether silage is an immunosuppressive and predisposes animals to infection is unclear (Gronstol, 1980b; Gitter *et al.*, 1986a). Wilesmith and Gitter (1986) suggested that the disproportionately high numbers of listeriosis incidents in Scotland may be simply due to the poor quality of silage produced as a result of the harsh Scottish weather conditions.

Since the organism is ubiquitous in the environment it is feasible that silage is commonly contaminated with *L. monocytogenes* at the grass cutting stage. Growth of the organism from the presumably low levels initially present is dependant upon the quality of fermentation. Within a few inches of the surface the aerobic environment provides suitable conditions for multiplication, whereas deeper

within the silage anaerobic conditions and fermentation of the natural sugars leads to acidification and an inhibitory environment (Fenlon, 1985). Welshimer (1960a) found that at 6°C the peak of logarithmic growth occurs in 10 to 11 days and the elapse of several months between silage making and its use allows ample time for considerable surface multiplication.

1.9.3 Serotyping of isolates

Serotyping of *L. monocytogenes* isolates is of limited value for epidemiological studies as the majority of pathogenic strains belong to serovars 1/2a, 1/2b, or 4b (Seeliger, 1961; Gray and Killinger, 1966; Weis, 1975b; Kampelmacher and van Noorle Jansen, 1979; Ralovich *et al.*, 1986; Audurier *et al.*, 1986). In Norway an equal distribution of serogroups 1 and 4 were isolated from ovine and caprine encephalitis cases but only serogroup 1 strains were isolated from caprine abortion material (Kummeneje, 1975). Serotype 1/2 was reported as the dominant isolate from animals in Hungary (Ralovich *et al.*, 1986). Similarly in Great Britain between 1981 and 1984 one hundred and twenty six isolates from sheep belonged to serotype 1/2, forty one to serotype 4 and nineteen to serotype 5 (Audurier *et al.*, 1986). These authors report the identification of different serotypes and phage types from single flocks and suggest the possibility of different sources of infection

existing in single outbreaks. The prevalence of a limited number of serovars of *Listeria* means identification of epidemiologically related strains is impossible by serotyping alone and alternative typing systems have been sought.

1.9.4 Phagetyping

Bacteriophages classified as belonging to the *Myoviridae* and *Siphoviridae* (Rocourt, 1986) have been identified for all five *Listeria* species (Rocourt *et al.*, 1985a; Rocourt, 1986; Rocourt *et al.*, 1986). Since in general the lytic spectrum of the phage is restricted to strains of the parent serovar, phage receptors may be related to somatic antigens (Audurier *et al.*, 1984). Phage typing systems have been shown to be of value in epidemiological studies (Audurier *et al.*, 1984; McLauchlin *et al.*, 1986a) and a number of international centres have been established (Rocourt *et al.*, 1985b). However, these centres are restricted and as approximately 50% of serotype 1/2 strains cannot be phagetyped the method has not received wide application in veterinary fields.

1.9.5 Other systems.

Biotyping has been briefly described (Seeliger, 1961) but appears to have received virtually no application in epidemiological studies.

1.10 Host resistance to *Listeria*

1.10.1.1 Immune response

Listeria monocytogenes is capable of multiplication within cells of the monocyte macrophage series (Mackaness, 1962; Armstrong and Sword, 1964; 1966) and has been extensively used in studies of the murine immune response to intracellular parasites. Though this section briefly summarises important aspects of the immune response to *Listeria* the articles by: Hahn and Kaufmann (1981); Kaufmann (1984a; 1984b); Bortolussi *et al.* (1984); Kaufmann (1985) are recommended for a thorough review and a better understanding of cell-mediated immunity, macrophage function and host defence.

The classical work of Mackaness has established the importance of the monocyte macrophage and thymus derived (T-cell) lymphocyte in the host's immune response to *L. monocytogenes*. In naive mice following the intravenous injection of *L. monocytogenes* resident macrophages in the

liver and spleen capture approximately ninety per cent of the inoculum within ten minutes. Two hours later as few as fifty per cent of the bacteria remain viable. The surviving organisms undergo logarithmic intracellular growth with a peak in numbers at two or three days where upon acquired cellular resistance, marked by massive accumulation of macrophages in the liver and spleen, leads to rapid bacterial inactivation (Mackaness, 1962; Lane and Unanue, 1972; Mitsuyama *et al.*, 1978). This period of acquired resistance to *Listeria* lasts approximately three weeks (Mackaness, 1962) though the degree of protection is dependant on the challenge dose (Lane and Unanue, 1972).

The concept of cell-mediated immunity has arisen because protection against intracellular pathogens (Table 1.5) is antigen specific and can be adoptively transferred to nonimmune mice with lymphocytes or spleen cells from immune donors (Mackaness, 1969; Lane and Unanue, 1972; North, 1975). Conversely no protection is offered by the passive transfer of hyperimmune serum (Mackaness, 1962; 1969; Miki and Mackaness, 1964). Peak protection against *L. monocytogenes* is achieved with cells taken 6 or 7 days after infection and protection is proportional to the numbers of cells infused. Maximum expression is dependant upon the viability of spleen cells and on their ability to replicate (Mackaness, 1969). Protection is totally abrogated by prior treatment of lymphocytes with anti-

thymocyte serum (Lane and Unanue, 1972) or cyclosporin A (Strauss *et al.*, 1985).

Sensitized T-cell lymphocytes are produced as part of the animal's immune response but protection requires the mediation of monocyte macrophages (Mackaness, 1969; Lane and Unanue, 1972). Suppression of bacterial growth in the liver has been shown to be inversely related to the accumulation of macrophages which may arise from local proliferation or infiltration of circulating blood cells. Infiltration of monocytes is presumed to be of greater importance as X-ray irradiation of the liver, which destroys the proliferation of resident macrophages, does not reduce the host's immune response (North, 1970).

Table 1.5.

Facultative intracellular bacteria

(according to Hahn and Kaufmann, 1981)

Brucella species
Erysipelothrix rhusiopathiae
Francisella tularensis
Legionella pneumophila
Listeria monocytogenes
Mycobacterium leprae
Mycobacterium tuberculosis
Salmonella paratyphi
Salmonella typhi
Shigella flexneri
Treponema pallidum
Yersinia species

Vice versa whole body irradiation with screening of the liver results in an abrogation of the immune response (Mitsuyama *et al.*, 1978). The "activated" macrophages have markedly bactericidal properties both *in vivo* and *in vitro* (Mackaness, 1962; Miki and Mackaness, 1964), are larger, spread faster on glass surfaces and have more mitochondria and lysosomes (Mackaness, 1969; Hahn and Kaufmann, 1981).

The activated macrophage is a nonspecific effector cell not directed solely against the infecting organism. Peritoneal macrophages from mice immunised with *Salmonella typhosa* (*sic*) suppress the *in vitro* multiplication of *L. monocytogenes* (Armstrong and Sword, 1964). After intravenous challenge with *Brucella abortus* there is a short period when murine peritoneal macrophages are capable of *in vitro* inactivation of heterologous organisms and convalescent animals are also resistant to *in vivo* challenge by *L. monocytogenes* (Mackaness, 1964). Conversely the immune response of T-cells is antigen specific as the period of nonspecific resistance can only be recalled by challenge with homologous organisms (Mackaness, 1964) and protection against *L. monocytogenes* induced by live Bacillus Calmette-Guerin (BCG) organisms cannot be adoptively transferred to recipients (Mackaness, 1969).

Though Wirsing von Koenig *et al.* (1983) report intravenous injection of 10^9 c.f.u. *L. innocua* results in slight protection of mice against a secondary challenge by *L. monocytogenes*, no examination was made for acquisition of delayed type hypersensitivity (DTH) or for the presence of immunospecific T-cells. These results may be attributable to nonspecific enhancement of macrophage function. Kaufmann (1984b) categorised *L. monocytogenes* into persistent strains which following intravenous inoculation survive in the liver for at least four days or nonpersistent strains which are rapidly destroyed. Only persistent strains stimulate marked T-cell responses as measured *in vitro* by antigen induced proliferation and interleukin production, or *in vivo* by induction of protection and development of a DTH response.

Intracellular growth is a prerequisite for the development of cell-mediated immunity as no protection against intravenous challenge is provided by high doses of nonpersistent strains (Kaufmann, 1984b) or repeated injection of a nonhaemolytic avirulent mutant (Berche *et al.*, 1987a). Further, antibiotic abridgement of listeric infection results in a significant reduction in the production of protective T-cells (North *et al.*, 1981). However, nonpersistent strains of *Listeria* do express epitopes shared with persistent strains since T-cell lymphocytes from immune mice respond to heat-killed

nonpersistent strains and these organisms can stimulate *in vitro* proliferation of antigen specific T-cell clones (Kaufmann, 1984b).

Mice remain highly resistant to a lethal challenge by *L. monocytogenes* for several months despite the loss of protective T-cells from the spleen three weeks after challenge (North, 1975). Limitation of secondary infection is antigen specific and therefore not mediated by activated macrophages. It is associated with a faster and more extensive production of protective lymphocytes than in primary infections and the organism only multiplies in tissues for 24 hours after challenge. Long term immunity is associated with persistence of DTH and depends on the presence of nonreplicating protective T-cells detected by their resistance to vinblastine and by their rate of tritium incorporation (North and Deissler, 1975). In rats, radiolabelling of these cells shows they are rapidly produced in response to primary infection and after transfer to recipients have a short circulating life-span (MacGregor *et al.*, 1971). They move into exudates induced by a variety of inflammatory stimuli (Koster *et al.*, 1971) and from these foci are highly capable of transferring immunity, being more effective than spleen or thoracic duct cells from immune rats or peritoneal exudate cells from normal rats (Koster *et al.*, 1971).

1.10.1.2 Delayed type hypersensitivity

The onset of immunity to intracellular organisms is coincidental with the acquisition of DTH to parasite derived antigens (Mackaness, 1962) and passive transfer of immune lymphoid cells results in acquisition of both protection and DTH (Mackaness, 1969). This relationship between DTH and cellular resistance suggests they are manifestations of the same immunological mechanism with both DTH and acquired resistance dependant upon immunospecific T-cells. Though Kaufmann and Hahn (1982) have described the adoptive transfer of both DTH and protection with a cloned T-cell line the use of double layer soft agar for cloning does not rule out the possibility of the cells being derived from more than a single progenitor. It remains controversial whether a single T-cell subset can mediate both DTH and protection (Bortolussi *et al.*, 1984).

1.10.1.3 MHC restriction of cell-mediated immunity

Cell-mediated immunity cannot be passively transferred to nonsyngeneic mice as T-cell lymphocytes recognise antigens presented by macrophages in association with membrane glycoproteins encoded in genes of the Major Histocompatibility Complex (MHC) (Zinkernagel *et al.*, 1977). In the mouse the MHC is referred to as the H-2

complex and within this the I locus codes for surface structures, or MHC class 2 molecules, which are expressed on macrophages, B-cell lymphocytes and some T-cell lymphocytes (Hahn and Kaufmann, 1981). Interaction between Ly 1^+ helper T-cells and antigen presenting macrophages requires direct contact and is dependant upon histocompatibility at the H-2I loci (Zinkernagel *et al.*, 1977; Farr *et al.*, 1979; Ziegler and Unanue, 1979). This interaction together with monocyte derived lymphocyte activating factor, or interleukin 1 (IL 1), leads to lymphocyte secretion of lymphokines including interleukin 2 (IL 2) (Kaufmann *et al.*, 1982a; 1982b) and gamma interferon. Gamma interferon increases the tumoricidal and antibacterial activity of macrophages and in mice has been shown to confer *in vivo* protection against systemic listeriosis (Kiderlen *et al.*, 1984).

1.10.1.4 Protection by specific T-cell subsets

The ability of different T-cell subsets to adoptively provide protection in mice has been extensively studied (Hahn and Kaufmann, 1981; Bortolussi *et al.*, 1984; Kaufmann, 1984a). *In vitro* a single population of Ly 1^+ helper T-cells undergo *Listeria* specific proliferation, interleukin secretion and *in vivo* can confer a DTH response to listeric antigens (Kaufmann and Hahn, 1982). However, large numbers are necessary for adoptive transfer of

protection suggesting that in natural infections there are T-cell interactions and a requirement for different subsets. Ly $2^{+}3^{+}$ cytotoxic T-cells recognise antigen associated with MHC class 1 molecules and with stimulation by exogenous IL 2 secrete gamma interferon and cause target cell cytolysis (Kaufmann *et al.*, 1986). The release of *Listeria* by the cytolysis of infected cells may not be detrimental to the host as cytolytic T-cells will only be active in infective foci in the presence of IL 2 and the presence of gamma interferon may allow activated macrophages to take up and destroy the released organisms. The role and relevance of T-cell subsets in listeric infections of ruminants is currently unknown.

1.10.2 Genetically determined resistance

Genetically determined differences in innate resistance to listeric infections exist among various inbred strains of mice (Cheers and McKenzie, 1978; Kongshavn *et al.*, 1980) with differences due to qualitative and quantitative variation in the accumulation and activity of monocyte macrophages (Kongshavn, 1985). These are phenotypic traits since macrophages transferred from resistant strains of mice behave as host macrophages in susceptible strains (Hahn and Kaufmann, 1981). Innate resistance is controlled by an autosomal, dominant, non H-2 linked gene (Sadarangani *et al.*, 1980; Stevenson *et al.*, 1981; Hahn and Kaufmann,

1981). Though the possibility of genetic variation in the innate resistance of ruminants exists its importance to listeric infection is currently unrecognised.

1.10.3 Immunosuppression of the host

In humans, age and pregnancy related defects in cell-mediated immunity have been described (Bortolussi *et al.*, 1984). This may explain the prevalence of listeric infections in neonates and during pregnancy. A pregnancy associated suppression of murine cell-mediated immunity is indicated by an increase in the numbers of viable *L. monocytogenes* in maternal liver and spleen beyond the third day after infection (Luft and Remington, 1982). Similarly in pregnant sheep mitogen induced proliferative responses of circulating lymphocytes are markedly impaired in comparison to those of lymphocytes from nonpregnant animals (Burrells *et al.*, 1978). In neonatal mice a reduced number of macrophages bearing MHC class 2 antigens and a failure of infected macrophages to produce IL 1 are associated with impaired T-cell activity and an inability to restrict the growth of *L. monocytogenes* (Lu and Unanue, 1982). Alpha-fetoprotein is present in mammalian fetal serum and may also suppress T-cell activity, particularly of Ly 1^+ helper T-cells (Luft and Remington, 1982). Though these studies indicate that immunosuppresion may be involved in listeric

infections its importance for ruminants has yet to be determined.

1.11 Vaccination

Since the organism is an intracellular parasite and cell-mediated immunity has been shown to be of importance in mice it is probable that killed vaccines will be ineffective in providing any protection. A live, attenuated vaccine developed in Bulgaria is available in some European countries (Ivanov *et al.*, 1979) but details of its composition are unavailable. This vaccine is claimed to be effective in sheep though the results of field trials are equivocal (Gudding *et al.*, 1985) and no experimental model of listeric encephalitis is available to test its potential. It seems that before a vaccine can be produced further investigation is necessary to determine mechanisms of immunity and to establish their role in the pathogenesis of encephalitic forms of the disease.

1.12 Diagnosis of listeric infections

Listeriosis can be diagnosed in the laboratory by: cultivation of the organism, demonstration of the infectious agent or its products in tissues or body fluids, detection of a specific immune response, or in the encephalitic form of the disease by demonstration of the pathognomonic histological lesions (*vide supra*).

1.12.1 Cultivation

Cultivation of the organism from a normally sterile site is usually relatively easy with growth readily occurring on simple laboratory media. However, primary isolation can be difficult where the organism is present in low numbers or from a heavily contaminated environment (Seeliger, 1961; Gray and Killinger, 1966). Isolation from brains is difficult (Biester and Schwarte, 1939; Gray *et al.*, 1948) but success rates can be improved either by macerating sections (Gill, 1933) or by using the "cold enrichment" technique (Gray *et al.*, 1948). The principle of the latter method is poorly understood. It may depend upon the organisms' ability to replicate at low temperature or upon release of the bacteria from an intracellular location (Seeliger, 1961). However, use of the technique for diagnosis is impracticable since successful isolation often requires cold enrichment for prolonged periods.

Selective media have been described for the isolation of *L. monocytogenes* from contaminated samples such as faeces, water, and human or animal feedstuffs (Kampelmacher and van Noorle Jansen, 1969; Watkins and Sleath, 1981; Fenlon, 1985; Doyle and Schoeni, 1986; Lovett *et al.*, 1987). The media contain a variety of agents inhibitory to the competitive flora and these may include: lithium chloride, nalidixic acid, potassium thiocyanate, acriflavine, thallous acetate, and cycloheximide. Doyle and Schoeni (1987) compared three selective procedures for the isolation of *L. monocytogenes* from cheese. The results showed the organism was often only isolated by one procedure and none of the methods was entirely reliable.

1.12.2 Demonstration of the organism

Direct demonstration of the organism in tissue sections or in body fluids has been described. Gram's stains are of little use other than in experimental studies as the organism may be present only in low numbers and identification is based solely on morphology. Fluorescent antibody techniques (Eveland, 1963; Biegeleisen, 1964; Watson and Eveland, 1965) and peroxidase anti-peroxidase staining with polyclonal sera (Domingo *et al.*, 1986) have been described. However, results from the use of polyclonal sera must be regarded with caution because of the likely abundance of cross-reactions with other

bacteria. *Listeria* specific monoclonal antibodies may be useful but their use has not yet been described.

1.12.3 Serodiagnosis

A variety of serodiagnostic techniques exist for the detection of an antibody response which is assumed to be a reflection of previous infection by *L. monocytogenes*. The serum agglutination test (Widal test) has been used most extensively though early studies were unsatisfactory since the complexity of the antigenic groups was unrecognised and a variety of methods for antigen preparation were used.

Standard serum agglutination techniques using heat-killed cell suspensions for the detection of antibodies to somatic (O) antigens and with formalin treated organisms for flagellar (H) antigens have been described (Seeliger, 1961; Larsen *et al.*, 1980). Difficulties with strains failing to agglutinate and problems of autoagglutination can be overcome respectively by trypsin treatment and sonication of antigen (Seeliger, 1961; Osebold *et al.*, 1965; Gray and Killinger, 1966). However, the major drawback with the serum agglutination test is its nonspecificity since antibodies to *L. monocytogenes* are frequently found in sera from healthy animals with no histories of listeric infections (Osebold and Sawyer, 1955; Seeliger, 1958; Seeliger, 1961; Osebold, 1965; Aalund *et al.*, 1966; Gray

and Killinger, 1966). These titres may arise from subclinical infection or since *L. monocytogenes* shares common antigens with a variety of Gram positive organisms, they may be the result of exposure to other bacteria (Seeliger, 1958; Welshimer, 1960b; Aalund *et al.*, 1966; Minden *et al.*, 1972; Larsen *et al.*, 1974).

Studies have indicated that nonspecific reactions in the assay are a feature of the IgM class of antibody and efforts to improve the specificity of the agglutination test have included the pretreatment of sera with 2-mercaptoethanol (Aalund *et al.*, 1966; Osebold and Aalund, 1968). However, as IgM class antibodies are important for agglutination their removal may reduce the sensitivity of the test. Indeed in an examination of sera from ten bacteriologically confirmed cases of listeriosis only three were positive after 2-mercaptoethanol reduction (Larsen *et al.*, 1974). Other efforts to improve specificity have included preabsorption of sera with *Staphylococcus aureus* or Lancefield's group D *Streptococci* (Seeliger, 1961). However the methods are cumbersome, the distinction between positive and negative results is arbitrary and since the sensitivity and specificity are in doubt interpretation of the findings is difficult (Seeliger, 1961; Larsen *et al.*, 1980). Serum agglutination tests are thus unsatisfactory for diagnosis and must be used with caution in epidemiological studies.

A complement fixation test has been described (Seeliger, 1961) though it appears to offer no advantage over the serum agglutination test and may be less sensitive (Potel, 1963). Haemagglutination and haemagglutination inhibition tests suffer from a lack of specificity since antigens which spontaneously adhere to erythrocytes have been demonstrated in *L. monocytogenes* (Neter *et al.*, 1960) and are common to a number of Gram positive organisms (Rantz *et al.*, 1956). A haemagglutination assay has been described (Gronstol, 1979a) in which erythrocytes are sensitized with the E_i extract from *L. monocytogenes*. However, this ether extracted preparation is a complex mixture which differs antigenically between strains (Mara *et al.*, 1974a). Although antibodies were detectable in the sera of animals after episodes of listeric abortion or septicaemia the assay has not been validated experimentally and low titres amongst healthy ewes are widespread (Gronstol, 1979a; 1979b; Loken and Gronstol, 1982).

Antibody precipitation has been shown with a variety of antigenic fractions in agar gel diffusion tests (Pease *et al.*, 1972; Jain and Chandiramani, 1978). The specificity of these crude antigen preparations is dubious and their practical use for serodiagnosis has not been described. Growth inhibition tests which detect antibodies to somatic antigens and agglutination immobilization assays which detect flagellar antigens have been reported (Potel, 1979;

Kuhlmann-Berger and Potel, 1985). However after vaccination of sheep with rough strains of *L. monocytogenes* only the agglutination immobilization assay detected an antibody response. In a study of 237 sera from four flocks only low titres were found and no interpretation of the single titres was possible. A comparison of an ELISA using whole organisms as antigen, complement fixation, and agglutination assays for the diagnosis of perinatal listeriosis has been made (Hudak *et al.*, 1984). The complement fixation assay was the best assay though its sensitivity was only 78 per cent and the positive predictive value 75 per cent. Though all the tests were relatively insensitive intriguingly the authors report they were useful in ruling out infection.

To date the use of crude antigens has been unrewarding and efforts have been made to find a *Listeria* specific antigen which is of use in serological tests. Peel (1987) used western blotting to examine for specific protein antigens but demonstrated extensive cross-reactions between *L. monocytogenes* cell wall antigens and sera from rabbits vaccinated with *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermis*, and *Streptococcus faecalis*. Conversely antigens of these organisms were recognised by antisera raised in a specific pathogen free lamb after vaccination with *L. monocytogenes*.

Affinity purified flagellin of *L. monocytogenes* serovar 4b is a specific antigen but results from an ELISA indicate that flagellin is not useful for serodiagnosis (Peel, 1987). Delvallez *et al.* (1979) identified a specific surface antigen of *Listeria*, "antigen 2". Studies have shown this to be common to the genus and to have a molecular mass of 17 kDa. Its potential in serological tests has been briefly described (Carlier *et al.*, 1980).

Ovine listeriosis: development of novel serological assays for diagnosis, experimental validation and field investigations into the epidemiology of infection.

Research objectives.

This work was funded by The Scottish Office Agriculture and Fisheries Department and its principle aim was the study of listeriosis in sheep. This condition is a considerable problem for sheep farmers, causing significant economic loss, widespread animal suffering and restricting the safe use of silage as a feedstuff. The study was carried out in collaboration with the Bacteriology Section of the Moredun Research Institute (Animal Diseases Research Association) and extended the research into the protein antigens of *Listeria monocytogenes* initiated by Drs. W. Donachie and Mary Peel.

The major research objectives of the study were:

1. to identify and isolate the somatic antigen determinants of *L. monocytogenes*.

2. to examine the clinical and serological responses of sheep to oral infection by the organism.

3. to develop a reliable serological assay and to undertake field investigations into the epidemiology of ovine listeriosis.

4. to establish a diagnostic test for listeric encephalitis.

The epidemiology and pathogenesis of ovine listeriosis are poorly understood. Though *L. monocytogenes* is widespread in the environment it is unknown how frequently animals are exposed to infection and at what dose clinical disease is likely to develop. A consideration of the previous literature review will show that the oral route is potentially important in the pathogenesis of listeric infections yet the clinical, pathological and serological responses of sheep to oral challenge have not been studied.

It is also apparent that despite extensive research over the past fifty years the immunological determinants of *L. monocytogenes* remain obscure. Current procedures for the detection of anti-*Listeria* antibodies using crude antigen preparations have been unrewarding and all suffer from a lack of sensitivity and specificity. Though a study was carried out into the protein antigens of *L. monocytogenes* (Peel, 1987) no antigen was identified which allowed the measurement of a serological response to infection. Delvallez *et al.* (1979) isolated a genus specific antigen and although an initial report suggested it was useful in a diagnostic assay (Carlier *et al.*, 1980) no subsequent studies have been described.

The development of a successful and precise immunological assay for listeric infection is dependant upon the examination of serological responses to defined antigens. It is envisaged that the production of monoclonal antibodies recognizing cell wall antigens will permit the definition, isolation and purification of the somatic antigen determinants of *Listeria monocytogenes*. In combination with the experimental challenges it is proposed to examine the serological response of animals to the defined virulence factor listeriolysin O (LLO) and to the somatic antigen determinants. These studies should allow the development of useful serological tests for application in diagnosis and epidemiological studies.

Materials and Methods

Chapter 2.

Materials and methods.

2.1 General materials and methods

2.1.1 Sterilization

Sterilization was carried out by: 1. autoclaving at 121°C, 15 p.s.i for 15 min, or 2. by membrane filtration using a 0.22 μm membrane filter (Millipore).

2.1.2 Aseptic techniques

Cell culture and microbiological techniques were carried out with aseptic techniques using initially sterile materials.

2.1.3 Phosphate buffered saline (PBS)

8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, were dissolved in 1 l of water to give a phosphate buffer of pH 7.4. Where necessary sterilization was performed by autoclaving. For haemolysin assays PBS was adjusted to pH 6.0 by the addition of 1M HCl.

2.1.4 Carbonate/Bicarbonate buffer, pH 9.6.

1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, were dissolved in 1 l of distilled water.

2.1.5 Gram's stain method

A small quantity of bacterial growth was emulsified in saline on a glass microscope slide, air dried and fixed by direct heat. The slide was flooded with 0.5% crystal violet for 1 min, then flooded with Lugol's iodine for a further minute. The slides were decolourized briefly with absolute alcohol and washed thoroughly with running tap water. Slides were counterstained with 1 in 10 carbol fuchsin for 30 sec, washed and blotted dry.

2.1.6 Bacterial viable counts

Viable counts of bacteria were determined by limiting dilution according to the method of Miles *et al.* (1938).

2.2 Bacterial strains

2.2.1 *Listeria* strains

Culture collection strains of *L. monocytogenes* serovar 1/2a (NCTC 7973), *L. monocytogenes* serovar 4b (NCTC 10527), *L. ivanovii* serovar 5 (SLCC 2379), *L. innocua* serovar 6a (NCTC 11288) together with the following wild strains of *L. monocytogenes* were used in this study. *Listeria monocytogenes* serovar 4b (L1059) isolated from a field case of caprine listeric encephalitis and serovar 1/2a (L72) isolated from a case of ovine listeric encephalitis.

The additional *Listeria* strains used in the study of the serovar specificity of the monoclonal antibodies (Chapter 3) were obtained from Dr. J. Rocourt, Pasteur Institute and Dr M. Peel, Moredun Research Institute.

Plate 2.1.

Appearance of *Listeria monocytogenes* serovar 4b (L1059) 18 h colonies growing on 5% sheep blood agar at 37°C.

Plate 2.2.

Morphology of *Listeria monocytogenes* serovar 4b (L1059) stained by Gram's method.

Plate 2.1.

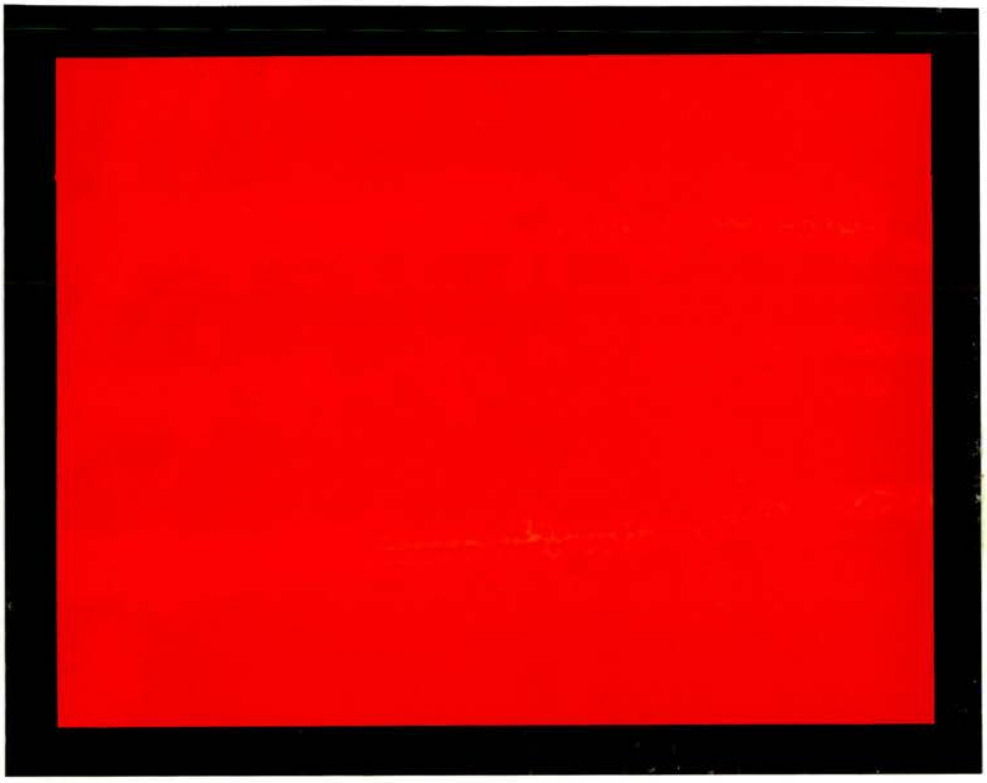
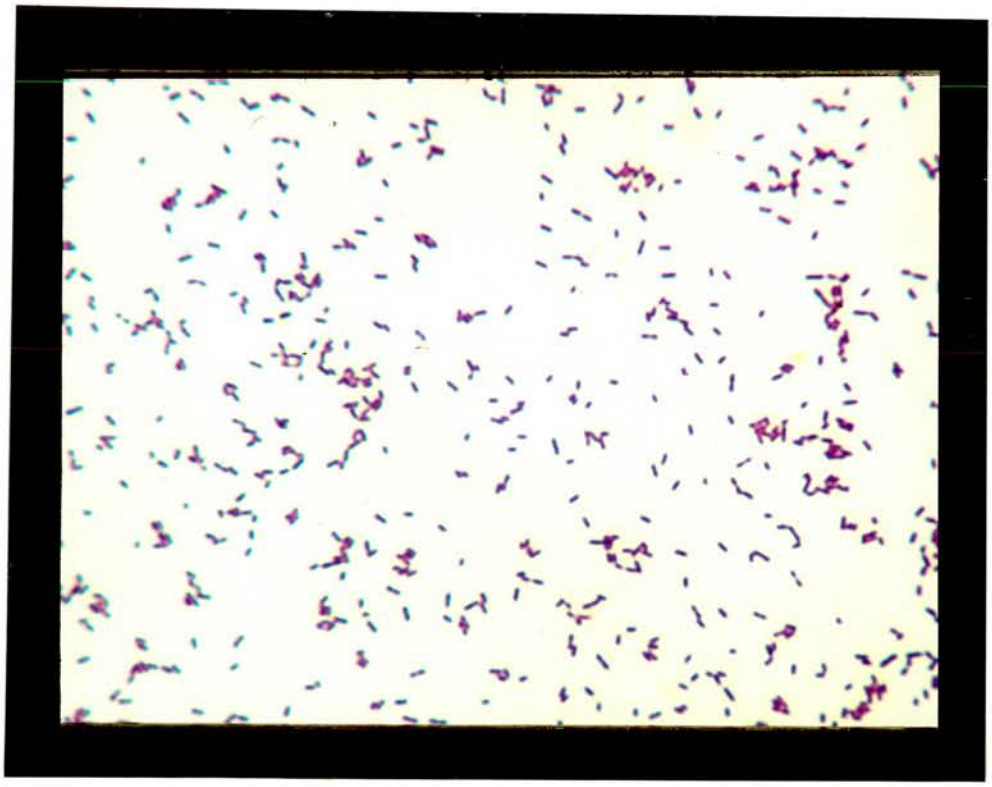


Plate 2.2.



2.2.2 Storage of bacterial strains

Strains of bacteria for experimental studies were stored lyophilized and on dorset egg slopes (Difco) at 4°C. Strains of bacteria received for identification and characterization were stored on nutrient agar slopes at 4°C.

2.3 Culture media

2.3.1 Blood agar with 5% sheep blood

Dehydrated blood agar base 40 g (Gibco) was suspended in 1 l of distilled water and sterilized by autoclaving. A 50 ml volume of sterile sheep blood, collected in 4% citrate, was added after cooling the medium to between 45°C and 50°C. The mixture was dispensed in 13 ml volumes into sterile petri dishes (Sterilin). Plates were dried prior to use and stored at 4°C.

2.3.2 Tryptic soy broth (TSB)

30 g of dehydrated TSB powder (Gibco) was dissolved in 1 l of distilled water, dispensed in bottles in the required volumes and sterilized by autoclaving.

2.3.3 Brain heart infusion broth (BHIB)

38 g of dehydrated BHIB powder (Gibco) or 37 g of Oxoid BHIB powder was dissolved in 1 l distilled water, dispensed in the required volumes in bottles and sterilized by autoclaving.

2.3.4 Tryptose phosphate broth (TPB)

29.5 g of TPB powder (Difco) was dissolved in 1 l of distilled water, dispensed in the required volumes in bottles and sterilized by autoclaving.

2.3.5 Listeria selective medium (Oxford formulation)

Oxford formulation agar was used as a selective and indicator medium for the isolation of *Listeria* species from faeces samples. 27.75 g of Listeria selective agar base (Oxoid) was suspended in 500 ml of distilled water and sterilized by autoclaving. One vial of reconstituted Listeria selective supplement (Oxoid) was added to the cooled medium, mixed well and the medium poured into sterile petri dishes. Plates were stored at 4^{o}C.

2.4 Identification of *Listeria* species

Isolates of *Listeria* spp. were identified using the criteria described by Rocourt *et al.* (1983). (Table 1.2).

2.4.1 Haemolysis on sheep blood agar

Cultures were streaked onto sheep blood agar plates, incubated at 37°C overnight and the areas adjacent to and below colonies examined for haemolysis.

2.4.2 Motility test

A drop from a six h to overnight BHIB culture, incubated at room temperature, was placed on the underside of a cover slip then suspended over a microscope slide on strips of blu-tack. Typical tumbling motility was examined for microscopically.

2.4.3 Catalase test

A drop from a six h to overnight BHIB culture, incubated at room temperature, was added to a drop of 10 volume hydrogen peroxide on a glass microscope slide. A cover slip was placed over the drop and a positive result was indicated by the production of gas bubbles on its undersurface.

2.4.4 Voges Proskauer test

The organism was grown in glucose phosphate broth at 37°C for 48 h. To 5 ml of culture 3 ml of 5% alpha-naphthol in ethanol was added followed by 1 ml of 40% KOH. After shaking a positive reaction was indicated by the development of a pink/orange colour.

2.4.5 Sugar utilization tests

L-rhamnose, D-xylose and alpha methyl mannoside (BDH) were stored at room temperature. 1.5 g of dehydrated Andrade's peptone water powder (Oxoid) was dissolved in 100 ml of distilled water and sterilized by autoclaving. 1 g of the test carbohydrate was added and the solution sterilized by filtration and aseptically dispensed in 3 ml volumes. Complete broths were stored at 4°C.

2.5 Animals

2.5.1 Animals (Scientific Procedures) Act 1986

All animals were housed and cared for by staff and stockmen of the clinical department MRI in accordance with Home Office regulations. All animal experiments and manipulations were licensed by: The Animals (Scientific Procedures) Act 1986.

2.5.2 Mice

Balb/C mice bred at MRI were used in these experiments. Mice were of either sex and four to six weeks old at the commencement of experiments.

2.5.3 Lambs

Six month old Suffolk cross Scottish Blackface lambs (Plate 2.3) were bred at Moredun Research Institute. The animals had never been fed silage and were housed in experimental groups in loose boxes with straw bedding. Feeding consisted of hay and concentrate (ESCA sheep nuts).

Plate 2.3.

Six month old Suffolk cross Scottish Blackface lambs typical of those used in the experiments.

2.6 Culture techniques

2.6.1 Dialysis sac cultures

Dialysis sac cultures were produced according to the principles described by Sutherland (1985).

2.6.2 Challenge inocula

Before the challenge experiments the bacteria were passaged through mice. Passage was effected by intraperitoneal inoculation of Swiss white mice with approximately $1x10^9$ c.f.u. *L. monocytogenes* obtained from 18 h BHIB (Oxoid) cultures. Bacteria were recovered from the livers of the mice 24 h after challenge. Challenge inocula were prepared by growing bacteria in 5 l of BHIB (Oxoid) for 18 h at 37°C. The organisms were pelleted by centrifugation (2,400 *g* for 1 h at 4°C) and washed three times in sterile PBS. The final bacterial pellets were resuspended in volumes of 25 ml PBS and 1 ml aliquots were maintained at -70°C until use. Viable counts of two aliquots were made prior to freezing and subsequently from one aliquot at the time of each challenge.

2.6.3 Method of culturing blood samples

Ten mls of venous blood were inoculated into Liquoid blood culture bottles (Roche) and these were incubated at 37°C for 48 h. After 24 and 48 h incubation subcultures were made onto sheep blood agar plates which were incubated for 48 h at 37°C and examined for bacterial growth. *Listeria* species were identified as previously described (section 2.4).

2.6.4 Method of culturing faeces samples

Faeces were cultured by homogenizing approximately 400 mg in 4 ml of sterile peptone water. 20 μl volumes were serially diluted in 2 ml of peptone water to give final dilutions of 10^{-3}, 10^{-5}, 10^{-7}, and 20 μl volumes of the four suspensions were plated in duplicate on *Listeria* selective medium (section 2.3.5). The plates were incubated for 48 h at 30°C and suspect colonies were subcultured to 5% sheep blood agar plates for identification.

2.6.5 Method for *in-vivo* growth of *L. monocytogenes*

A chamber was prepared by sealing the ends of a 14 cm length of silicone tubing (internal diameter of 1.9 cm) with 0.22 μm filter membranes (Millipore). The membranes were glued in place with silastic (Dow-Corning) and the chamber sterilized by autoclaving.

Listeria monocytogenes serovar 4b (NCTC 10527) was grown in 300 ml of TSB for 18 h at 37^{o}C. The bacterial pellet was collected and washed by repeated centrifugation (7,500 *g* for 30 min at 18^{o}C) and resuspension in sterile PBS. The final pellet, after three washes, was resuspended in sterile PBS at a concentration of $1x10^{10}$ c.f.u. ml^{-1}. 40 ml of the suspension was injected into the sterile chamber using a 19 gauge needle and the injection site was sealed with silastic. Using aseptic techniques the chamber was placed in the peritoneal cavity of an anaesthetized 18 month old goat. Three weeks later the animal was killed and blood was collected. After the formation of a blood clot the serum was collected and stored at -20^{o}C. The chamber was recovered, the contents were cultured and the purity of *L. monocytogenes* confirmed.

2.7 Preparation of antigens

2.7.1 Production of heat-killed *L. monocytogenes*

100 ml cultures of *L. monocytogenes* serovar 4b (NCTC 10527) were grown for 24 h at 37°C. Bottles containing 300 ml TSB were inoculated with 1 ml of the bacterial culture and incubated at 37°C for 18 h. The purity of the cells was confirmed by plating onto sheep blood agar plates and the bacteria were killed by exposure of the broths to moist heat at 100°C for 60 min. The broth cultures were centrifuged (5,000 *g* for 30 min at 4°C) and the bacterial pellets resuspended in distilled water. The cells were washed in the same manner three times and the final bacterial pellets lyophilized and stored at room temperature. Sterility was confirmed by culture of the killed broths and the final lyophilized products. Typically 300 ml culture broth produced 40 mg (dry weight) of bacterial cells.

Suspensions of heat-killed bacteria used in the indirect ELISA in section 3.1.2 and dot-blot assays in section 3.2.1 were produced as described above. The bacterial strains were: *L. monocytogenes* serovar 1/2a (NCTC 7973); *L. monocytogenes* serovar 4b (NCTC 10527); *L. ivanovii* serovar 5 (SLCC 2379); *L. innocua* serovar 6a (NCTC 11288).

2.7.2 Phenol extraction from whole *L. monocytogenes*

Cell wall non-covalently bound accessory lipocarbohydrates were extracted from whole *L. monocytogenes* cells using the phenol extraction method described by Heckels and Virji (1988).

A 5 l flask of TSB was inoculated with 10 ml from a 24 h culture of *L. monocytogenes* serovar 4b (NCTC 10527) grown at 37°C in TSB. After incubation at 37°C for 18 h with shaking the bacterial purity was ascertained by direct culture to blood agar plates. The bacterial cells were pelleted by centrifugation (2,400 *g* for 30 min at 4°C) and repeatedly centrifuged after resuspension in sterile PBS. After three washes 1.5 g (wet weight) of cells was resuspended in 2 ml of 0.1M sodium acetate, pH 4.5, buffer. Whole bacterial cells were defatted by mixing with 4 ml of absolute methanol and 2 ml of chloroform at room temperature overnight. The bacteria were recovered by filtration onto a 0.22 μm filter membrane (Millipore) and washed with 16 ml of absolute methanol.

The defatted cells were resuspended in 3 ml 0.1M sodium acetate buffer, pH 5, mixed with 3 ml of hot (60°C) 80% (w/v) aqueous phenol and left shaking in a water bath at 65°C for 45 min. After cooling in an ice bath the emulsion was broken into two phases by centrifugation

(4,100 *g* for 30 min at 4°C). The upper aqueous layer was removed by pipette and kept at 4°C. This layer was replaced with 2 ml 0.1M sodium acetate, pH 5, buffer and the centrifugation and collection of the aqueous layer repeated.

The two aqueous layers were combined and dialysed against 0.1M sodium acetate, pH 5, buffer at room temperature. The dialysis buffer volume of 200 ml was changed six times over 24 h. Nucleic acids were degraded by incubation of the dialysed extract at 15°C with 20 units of ribonuclease A (Sigma), 300 units deoxyribonuclease II (Sigma) and 40 μl 0.1M $MgCl_2$. Microbial contamination during the 24 h incubation was prevented by the addition of a small volume of toluene. The phenol extraction procedure was repeated to remove nuclease protein and the upper aqueous layer was collected and dialysed against 0.1M sodium acetate, pH 5, buffer at room temperature. The final aqueous extract of lipocarbohydrates (PE1) was brought to neutral pH by the addition of 1M Tris and stored at -20°C in 100 μl aliquots. A second extract of lipocarbohydrates, identified as PE2, was obtained in the same manner.

2.7.3 Cholesterol precipitation of listeriolysin O

LLO for immunoblotting was precipitated with cholesterol according to the method of Vazquez-Boland *et al.* (1989a). Briefly, *L. monocytogenes* serovar 4b (L1059) was grown in 5 l of BHIB (Oxoid) for 18 h at 37°C. The supernatant fluid was collected after centrifugation (2,400 *g* for 1 h at 4°C) and sterilized by filtration. 1 l of supernatant fluid was concentrated twenty fold by ultrafiltration at 4°C using an Amicon PM10 filter membrane (operating pressure 20 p.s.i.) and the retentate subject to ultracentrifugation (90,000 *g* for 60 min at 4°C). 20 ml of the concentrated supernatant fluid was diluted with 20 ml of 20mM L-cysteine in PBS, pH 6.0. Cholesterol (Sigma) dissolved in absolute alcohol (10 mg ml^{-1}) was added to give a final concentration of 500 μg cholesterol ml^{-1}. After incubation, with shaking, for 30 min at 37°C the precipitate was collected by centrifugation (25,000 *g* for 30 min at 4°C). This was washed three times by resuspension in PBS, pH 6, followed by centrifugation (25,000 *g* for 30 min at 4°C) and the final pellet was resuspended in 20 ml PBS, pH 7.4, and stored in 1 ml aliquots at -20°C.

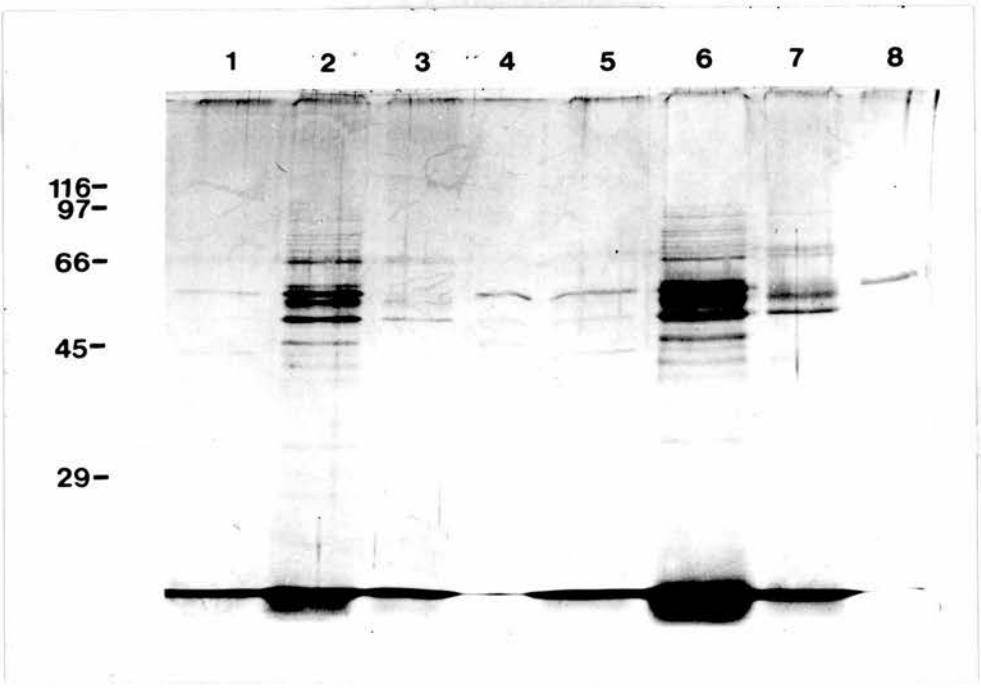
Figure 2.1.

SDS-PAGE analysis of the steps in the production of cholesterol precipitated LLO.

Lane 1, crude *L. monocytogenes* culture supernatant fluid (10 μl); lane 2, concentrated *L. monocytogenes* culture supernatant fluid (10 μl); lane 3, concentrated *L. monocytogenes* culture supernatant fluid after removal of cholesterol precipitate (10 μl); lane 4, cholesterol precipitate (10 μl); lane 5, crude *L. monocytogenes* culture supernatant fluid (20 μl); lane 6, concentrated *L. monocytogenes* culture supernatant fluid (20 μl); lane 7, concentrated *L. monocytogenes* culture supernatant fluid after removal of cholesterol precipitate (20 μl); lane 8, cholesterol precipitate (20 μl).

Position of molecular mass markers (kDa) shown.

Figure 2.1.



2.8 Reagents and techniques

2.8.1 Horseradish peroxidase conjugates

Species specific anti-IgG (heavy and light chain) antibodies conjugated with horseradish peroxidase were obtained from the Scottish Antibody Production Unit (SAPU). Conjugates were stored at -20°C in aliquots suitable for one assay.

2.8.2 Horseradish peroxidase reagents

2.8.2.1 Ortho-phenylenediamine dihydrochloride (OPD)

In ELISA the reagent in the substrate solution for horseradish peroxidase was OPD (Sigma). OPD was stored at -20°C. The substrate solution was made up immediately prior to use and consisted of 4 mg OPD plus 4 μl, 100 volume hydrogen peroxide in 2.43 ml 0.1M citric acid, 2.57 ml 0.2M sodium hydrogen phosphate and 5 ml distilled water.

2.8.2.2 Diaminobenzidine tetrahydrochloride (DAB)

DAB (Sigma) was used as the substrate reagent for horseradish peroxidase in dot-blot and immunoblotting studies. 5 mg of DAB dissolved in 1 ml Tris-HCl, pH 7.4, was stored at -20°C. Immediately prior to use 1 ml of DAB solution was added to 19 ml of Tris-HCl, pH 7.4, with 20 µl 100 volume hydrogen peroxide. This substrate solution was applied to the membranes and the colour allowed to develop at room temperature. The reaction was halted by rinsing in tap water.

2.8.3 ELISA techniques

The method originally described by Engvall and Perlmann (1972) was the basis for these assays.

2.8.3.1 Indirect ELISA to measure antibody to whole heat-killed cells of *L. monocytogenes*

Heat-killed *L. monocytogenes* serovar 4b (section 2.7.1) at concentrations of 80 µg (dry weight) ml^{-1} in PBS was coated onto Dynatech M129A microtitre plates at 100 µl per well (equivalent to 3.8 µg protein per well). Open plates were dried overnight at 37°C and before use subjected to heating in a microwave oven (Phillips, Cooktronic 7910) for ten min. Wells were washed five

times with wash buffer (PBS plus 0.05% (v/v) Tween 20) before blocking with wash buffer plus 4% (w/v) skimmed milk powder and left covered for 90 min at 37°C. The standard positive control antiserum was used at a fixed dilution of 1/100. The test serum samples diluted 1/100 in serum buffer (wash buffer plus 1% (w/v) skimmed milk powder) were added in 100 µl volumes to duplicate wells and the sealed plates incubated at 37°C for 90 min. Plates were washed as before and 100 µl donkey anti-sheep HRP conjugate (section 2.8.1) at a previously determined optimal dilution of 1/200 in serum buffer was added to all wells. Sealed plates were again incubated for 90 min at 37°C before washing as described. Finally 100 µl of substrate (section 2.8.2.1) was added to all wells. After 15 min at room temperature the reaction was stopped by the addition of 50 µl, 2.3M sulphuric acid. The optical densities at 492 nm were determined on a Titertek multiscan plate reader (Flow Laboratories) and the results for the test sera were expressed as the "percentage absorbance" of the positive standard serum (de Savigny and Voller, 1980).

$$\text{"percentage absorbance"} = \frac{\text{mean O.D. of test serum}}{\text{mean O.D. of standard}} \times 100$$

2.8.3.2 Competitive sandwich ELISA to measure antibody to the putative LTA

Serological responses of lambs to putative LTA were measured by competitive sandwich ELISA utilizing monoclonal antibodies produced in Chapter 3.

Purified mouse mAb 25/25 (ascitic fluid, section 3.4) was diluted to 50 μg protein ml^{-1} in sterile PBS and 100 μl added to wells of a 96 well flat-bottomed microtitre plate. Plates were left overnight at 4°C before washing and then addition of 100 μl of 5% (w/v) skim milk powder in PBS to all wells. After incubation, then thorough washing, excess antigen in the form of 100 μl of a 1/800 dilution PE2 (section 2.7.2) in serum buffer was added to all wells, except row 1 which received serum buffer alone. Plates were incubated for 90 min at 37°C before thorough washing. Meanwhile neat serum samples were diluted in equal volumes with a 1/200 dilution of HRP conjugated mAb 25/25 in serum buffer. Test and control sera were incubated at 37°C for 30 min before 100 μl volumes were added to paired wells. After incubation at 37°C for 90 min the assay was continued by washing and addition of substrate as described in section 2.8.3.1. The positive control was 10 μg of the unconjugated mAb 25/25 incubated with a 1/400 dilution of the HRP

conjugated mAb 25/25. The standard was a 1/400 dilution of HRP conjugated mAb 25/25.

The optical densities at 492 nm were determined on a Titertek multiscan plate reader (Flow Laboratories) and the results were expressed as the percentage inhibition derived from the degree of inhibition of the standard serum by the test as a percentage of the inhibition produced by the positive control:

$$\text{"percentage inhibition"} = \frac{\text{mean reduction of O.D. of standard by test serum}}{\text{mean reduction of O.D. of standard by positive control}} \times 100$$

2.8.4 Dot-blot technique

Dot-blots were performed using the technique of Sternberg and Jeppesen (1983). Dissolved or suspended bacterial antigens were immobilized as spots on nitrocellulose membranes (Schleicher and Schuell). These were dried at 37°C for 30 min then washed in distilled water and free binding sites blocked by incubation for 60 min at 37°C in horse serum (Gibco) diluted 1:1 in BWB (0.35M sodium chloride, 1mM EDTA, and 0.005% (v/v) Tween 20 in PBS). After blocking, membranes were probed with test sera diluted in serum diluent (BWB plus 10% (v/v) horse serum). Sera were incubated for 2 h at 37°C and then the membranes thoroughly washed in BWB. Membranes were incubated for 2 h at 37°C in the relevant anti-species HRP conjugates (section 2.8.1) at a dilution of 1/200 in serum diluent before washing thoroughly in BWB with a final rinse in 0.1M Tris-HCl, pH 7.4. Substrate (section 2.8.2.2) was added and reactions allowed to proceed until there was adequate colour development when the reaction was halted by washing in tap water.

2.8.5 Serum agglutination test

Serum agglutination tests were carried out using *L. monocytogenes* serovar 4b somatic antigen (Behringwerke AG). Geometric series dilutions of the test and control sera were made in PBS. The *Listeria* somatic antigen was added as 50 μl volumes to duplicate 50 μl volumes of the serum dilutions in U bottomed 96 well microtitre plates, the preparations were mixed well and left covered at 37^{o}C for 21 h. Agglutination was read visually against a dark background and the titre was recorded as the reciprocal of the final dilution showing visible agglutination.

2.8.6 Enzymatic digestion of bacterial proteins

Enzymatic digestion of bacterial proteins was carried out by protease treatment of whole bacterial cells. A 200 μl suspension of washed *L. monocytogenes* serovar 4b (NCTC 10527) containing approximately 320 μg protein was incubated for 60 min at 37^{o}C with 10 μl of Proteinase K (Sigma) containing 100 units protease ml^{-1} of buffer (0.01M Tris-HCl, pH 7.4). Protease enzymes were then destroyed by boiling at 100^{o}C for 30 min. The bacterial suspension was then incubated with 10 μl of Pronase E (Sigma) containing 40 units protease ml^{-1} of buffer (0.01M Tris-HCl, pH 7.4) at 37^{o}C for 60 min. Protease enzymes were destroyed by boiling at 100^{o}C for 30 min in

a water bath. To provide a control a bacterial suspension was included in the same procedure but without the addition of protease enzymes. Both bacterial suspensions were stored at -20°C.

2.8.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The procedure for SDS-PAGE was based upon the method of Laemmli (1970) as described by Hames (1987). The details were as follows:

2.8.7.1 Composition of buffers and reagents

Resolving gel buffer: 0.375M Tris-HCl, pH 8.8. Stored at 4°C.

Stacking gel buffer: 0.125M Tris-HCl, pH 6.8. Stored at 4°C.

Acrylamide stock solution: 30% (w/v) acrylamide (Bio-Rad), 0.8% (w/v) bisacrylamide (Bio-Rad) in distilled water. Stored at 4°C.

Electrode buffer: 0.025M Tris, 0.192M Glycine, 0.1% (w/v) SDS, pH 8.3. Stored at room temperature.

Sample buffer (double strength), pH 6.8: 1 ml stacking gel buffer, 1 ml distilled water, 1.6 ml 10% (w/v) SDS, 0.4 ml ß-mercaptoethanol (Sigma), 0.8 g glucose, 0.004% (w/v) bromophenol blue. Stored at 4°C.

Ammonium persulphate (APS) 1.5% (w/v): 30 mg APS was dissolved in 2 ml distilled water. Fresh solutions were made just before use.

TEMED: was used as supplied by Bio-Rad and stored at 4°C. TEMED catalyses the formation of free radicles from persulphate and these in turn initiate polymerization. The TEMED was therefore added to the gel mixtures immediately prior to pouring.

Sodium dodecyl sulphate (SDS) 10% (w/v): 1 g SDS was dissolved in 10 ml distilled water. The solution was stored at room temperature.

Molecular mass markers for SDS-PAGE

The molecular masses marked on the figures in this thesis were derived from the position of defined protein molecular mass markers obtained from Sigma (SDS-200). Specific molecular masses were calculated by reference to a standard curve derived from the relative mobilities of the standard proteins against the log10 of their molecular masses (Hames, 1987).

2.8.7.2 Composition of polyacrylamide gels

Different acrylamide concentrations in the polyacrylamide gels were acheived by using the recipes shown in Tables 2.1. and 2.2.

Table 2.1.

Recipes for resolving gel preparation

Stock solution (volume in ml)	Final acrylamide concentration in the resolving gel		
	15%	12.5%	10%
Acrylamide-bisacrylamide	10	8.3	6.7
Resolving gel buffer	2.5	2.5	2.5
10% SDS	0.2	0.2	0.2
1.5% APS	1.0	1.0	1.0
Distilled water	6.3	8.0	9.6
TEMED	15μl	15μl	15μl

Table 2.2.

Stacking gel recipe

Stock solution (volume in ml)	Final acrylamide concentration 3.75%
Acrylamide-bisacrylamide	1.25
Stacking gel buffer	2.5
10% SDS	0.1
1.5% APS	0.5
Distilled water	5.65
TEMED	10μl

2.8.7.3 Apparatus

Analytical protein electrophoresis was carried out using Protean I or mini Protean II vertical electrophoresis cells (Bio-Rad). (Plate 2.4).

Plate 2.4.

A mini Protean II vertical electrophoresis cell.

Plate 2.5.

A mini Protean II multiscreen apparatus.

Plate 2.4.

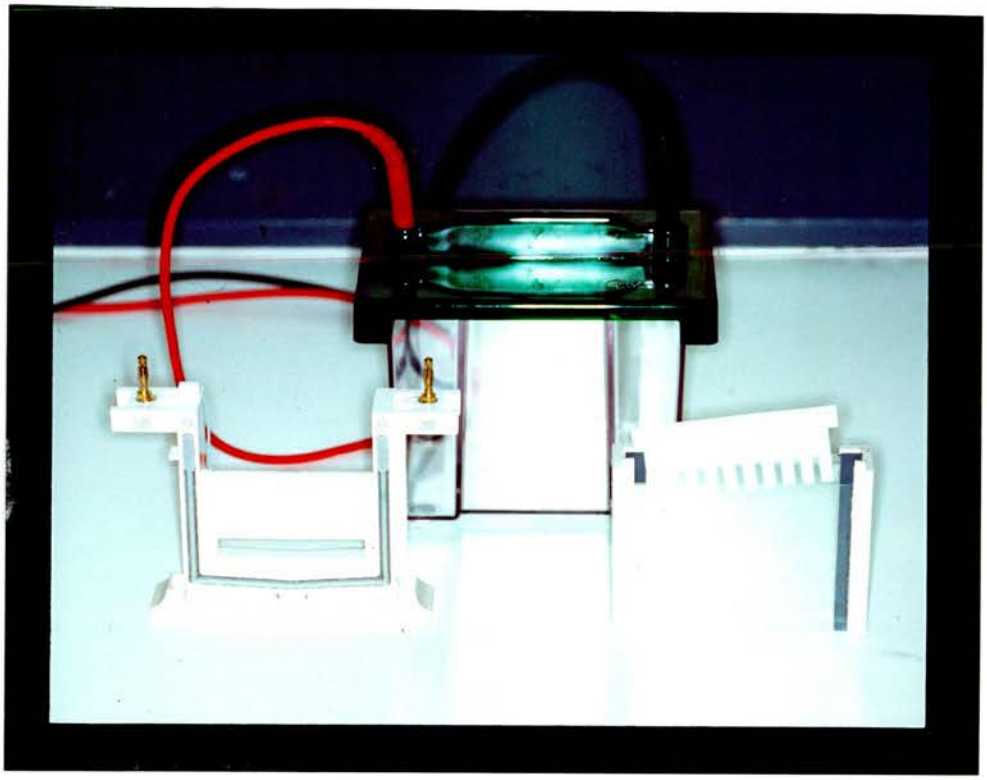
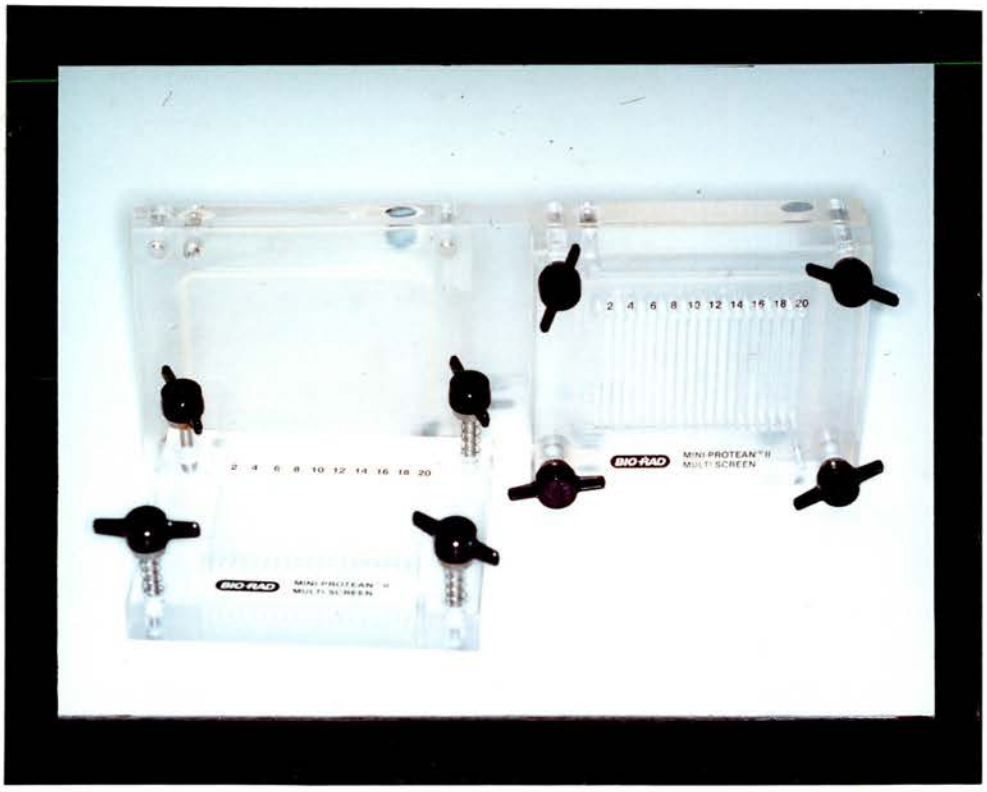


Plate 2.5.



2.8.7.4 Pouring gels, sample preparation and electrophoresis

The appropriate resolving gel mixture was degassed using a pump and the correct volume of TEMED added. The gel mixture was poured slowly between two clean glass plates using a pipette and overlaid with water saturated butanol. After polymerization the water saturated butanol was removed and the gel surface washed with a small volume of stacking gel buffer. The stacking gel mixture, prepared in the same manner as the resolving gel mixture, was poured on top. Without trapping air bubbles a comb forming single or multiple wells was immediately inserted in the stacking gel mixture and the assembly left undisturbed to allow polymerization to occur. After polymerization the gel was used immediately. Polymerized resolving gels, without stacking gels, were stored overnight at 4^{o}C overlaid with resolving gel buffer and sealed in cling film.

Samples were prepared for electrophoresis by immersion for 3 min, mixed 1:1 with double strength sample buffer, in a boiling water bath. Samples were then applied to the wells in the stacking gel, filled with electrode buffer, using a microsyringe. Electrophoresis was carried out at room temperature with a constant current of 40 mA.

2.8.8 Methods for silver staining of SDS-PAGE gels

Silver staining of polyacrylamide gels was performed either according to the method of Tsai and Frasch (1982) or Morrissey (1981).

2.8.9 Method for PAS staining of SDS-PAGE gels

Periodic acid-Schiff staining of carbohydrates in polyacrylamide gels was carried out by the method of Zacharius *et al.* (1969) after overnight fixation in 20% methanol, 10% acetic acid. Schiff's reagent was obtained from Sigma.

2.8.10 Immunoblotting

Examination of serological responses and characterization of monoclonal antibodies was carried out by immunoblotting as described by Towbin *et al.* (1979). After SDS-PAGE, electrophoretic transfer of proteins or lipocarbohydrates to Immobilon-P membranes (Millipore) or nitrocellulose membranes (Schleicher and Schuell) was achieved in transfer buffer (0.025M Tris, 0.2M Glycine, and 25% (v/v) aqueous methanol, pH 8.3) at 35 V and 10 mA for 16 h. Electrophoretic transfer was carried out in a Trans-blot cell (Bio-Rad) at room temperature or in a mini Trans-blot cell (Bio-Rad) with ice cooling.

In order to ascertain transfer had taken place and to establish the position of molecular mass markers the membranes were stained with a 0.2% (w/v) Ponceau S solution (Sigma). The membranes were washed in distilled water and free binding sites blocked by incubation for 60 min at 37°C in horse serum (Gibco) diluted 1:1 in BWB. Membranes were then probed with test sera diluted in serum diluent (section 2.8.4). Sera were either applied to numbered strips cut from the membranes or placed in rows on the membrane using a mini protean II multiscreen apparatus (Bio-Rad) (Plate 2.5). Sera were incubated for 2 h at 37°C, unless otherwise stated, then thoroughly washed in BWB and the assays continued as in section 2.8.4.

2.8.11 Passive haemagglutination assay

Using mouse monoclonal antibodies passive haemagglutination of sheep erythrocytes sensitized with a phenol extract of *L. monocytogenes* was demonstrated according to the method of Antonissen *et al.* (1981).

A 10% concentration of washed sheep erythrocytes was resuspended in PBS and mixed in equal volumes with 1/10, 1/100, or 1/1000 dilutions of PE2 (section 2.7.2) in PBS. Washed sheep erythrocytes were also resuspended to a final concentration of 5% in PBS. These erythrocyte suspensions were incubated at 37°C for 30 min then pelleted by centrifugation and resuspended first in 0.85% saline and then twice in PBS. The final erythrocyte concentration was 1% in PBS.

Control and test antibodies were double diluted in PBS in U bottomed 96 well microtitre plates (Sterilin Limited) and 50 µl volumes of each preparation of PE2 pretreated erythrocytes added to each antibody dilution. Plate contents were thoroughly mixed by shaking and left at room temperature overnight.

2.8.12 Haemolysin assay

Haemolytic activity in *L. monocytogenes* broth culture supernatant fluid and in chromatography column fractions was determined according to the method of Kreft *et al.* (1989). Briefly 200 µl of test samples were double diluted in 100 µl PBS, pH 6.0, in U bottomed 96 well microtitre plates and 10 µl of 0.1M dithioerythritol (DTE, Sigma) was added to all wells. Plates were covered and incubated on an orbital shaker for ten min at 37°C

before 10 μl of a 10% suspension of washed sheep erythrocytes in PBS, pH 6.0, was added to all wells and mixed thoroughly. Plates were covered and incubated for 30 min at 37^{o}C then cells were allowed to settle overnight at 4^{o}C. One haemolytic unit (HU) was defined as the reciprocal of the dilution which gave 50 per cent haemolysis. Positive control wells consisted of 10 μl 10% washed sheep erythrocytes in 100 μl distilled water with 10 μl 0.1M DTE. Negative control wells contained 10 μl of washed sheep erythrocytes in 100 μl PBS, pH 6.0, with 10 μl 0.1M DTE.

2.8.13 Protein assays

Protein assays were performed with the BCA enhanced protein assay (Pierce Chemical Company) using albumin fraction V (Pierce Chemical Company) as standard.

Protein concentrations were also estimated from the absorbance at 280 nm measured in quartz cuvettes in a Cecil SP6 spectrophotometer. The concentration of mouse antibody was calculated from the formulae:

$$\text{IgG (mg protein ml}^{-1}\text{ of antibody)} = \frac{\text{O.D. 280 nm}}{1.4}$$

$$\text{IgM (mg protein ml}^{-1}\text{ of antibody)} = \frac{\text{O.D. 280 nm}}{1.1}$$

2.8.14 Carbohydrate assays

Carbohydrate assays were performed using the method of Dubois *et al.* (1956) with glucose as standard.

2.8.15 Oral challenge technique

All oral doses were deposited through tubing to the back of the lambs' throats.

2.8.16 Histopathological techniques

The following tissues were collected into phosphate buffered neutral 10 per cent formalin: brain, spinal cord, spleen, kidney and liver. Blocks of these tissues, 3 mm thick, were prepared, dehydrated in alcohol, embedded in wax, sectioned at 4 μm and stained with haematoxylin and eosin. Coronal slices of the brains were taken from the forebrain at the level of the optic tract, from the midbrain at the level of the lateral geniculate body, from the medulla at the level of the inferior cerebellar peduncle and from the anterior cervical spinal cord. Sagittal sections were taken from the cerebellum.

2.9 Antibody production and purification

2.9.1 Production of ascites in mice and purification of monoclonal antibodies

Twenty two hybridoma cell lines secreting anti-*Listeria* antibodies were produced according to the method described in Chapter 3 (section 3.1.1). Adult Balb/c female mice were inoculated intraperitoneally with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane. Sigma). Three days later 10^7 cells from the required hybridoma cell line were inoculated intraperitoneally. Ascitic fluid was collected over the following days by inserting a 23 gauge needle into the peritoneal cavity and allowing the fluid to drain into a sterile bijou bottle. The fluid was clarified by centrifugation and stored at -20°C.

Monoclonal antibody was purified from ascitic fluid raised in mice using caprylic acid according to the method of McKinney and Parkinson (1987).

2.9.2 Production of an anti-*Listeria* hyperimmune antiserum

Listeria monocytogenes serovar 4b (NCTC 10527) was grown in 100 ml of TSB for 18 h at 37°C. The bacterial pellet was collected and washed by repeated centrifugation (7,500 *g* for 30 min at 18°C) and resuspension in sterile PBS. The final pellet, after three washes, was resuspended in 10 ml of 0.5% formol saline at a concentration of $5x10^8$ c.f.u. ml^{-1}. After incubation for 3 h at 37°C the bacteria were collected by centrifugation (5,000 *g* for 20 min at 18°C) and resuspended in 25 ml 0.5% formol saline. The vaccine was prepared by emulsifying equal volumes of the bacterial cell suspension in bayol/arlacell (90% bayol (ESSO), 10% arlacel (Sigma)). The final vaccine contained approximately 2 mg wet weight of bacterial cells ml^{-1}.

Two adult New Zealand White rabbits with no detectable antibodies to *L. monocytogenes* were immunized by repeated intramuscular injection of the vaccine according to the protocol described by Ullmann and Cameron (1969). One week after the final inoculation both rabbits were killed and serum was collected from the blood. The antiserum was stored at -20°C.

2.9.3 Production of a convalescent goat serum

The convalescent serum was collected from an 18 month old goat following the *in-vivo* growth of *L. monocytogenes* as described in section 2.6.5. The serum was stored at -20°C.

2.9.4 Rabbit anti-LLO antiserum

A rabbit polyclonal anti-LLO antiserum was kindly provided by A. Haas, University of Wurzburg. The hyperimmune antiserum was stored at -20°C.

2.9.5 Anti-isotype antibodies

Murine anti-sheep IgG_1 and IgM antibodies were kindly provided by Hoechst UK Ltd.

Laboratory Studies

Chapter 3.

Characterization of monoclonal antibodies raised against *Listeria monocytogenes* serovar 4b and an examination of their specificity for the organism.

Introduction

Diagnosis and epidemiological investigations are severely restricted by the non-specific and insensitive nature of the serological tests currently available (Osebold and Sawyer, 1955; Seeliger, 1958; Seeliger, 1961; Berche *et al.*, 1990; Low and Donachie, 1991) and the development of any useful serological assay for listeriosis depends upon the identification and isolation of specific antigens. Although considerable biochemical data is available on the structure of the cell wall of *Listeria monocytogenes* the exact nature of the somatic antigen determinants of the serological classification system remains unknown (Fiedler *et al.*, 1984; Fiedler and Ruhland, 1987; Ruhland and Fiedler, 1987). A previous study focussed on the protein antigens of *Listeria* spp. (Peel, 1987) but failed to identify serotype specific somatic antigens and a consideration of the previous literature review reveals substantial evidence for the somatic antigen determinants being polysaccharide in nature.

Since heat-killed bacteria are recommended for the production of antibodies to *Listeria* somatic antigens it was envisaged that the production of monoclonal antibodies (mAbs) to heat-killed *L. monocytogenes* would allow the recognition of specific somatic antigens and these antibodies would be of value in the isolation of antigens by methods such as affinity chromatography (Staehelin *et al.*, 1981). Additionally these antibodies could be used for the development of immunoassays to measure specific antibody levels by sandwich or capture ELISA (Hancock and Poxton, 1988; Shankarappa *et al.*, 1989).

Though mAbs to *L. monocytogenes* antigens have been described these either recognise flagellar antigens (Farber and Spiers, 1987a; Peel *et al.*, 1988b) and are reportedly ineffective for serodiagnosis (Peel, 1987; Low and Donachie, 1991) or they recognise uncharacterized antigens and their serodiagnostic potential has not been explored (Ziegler and Orlin, 1984). It was therefore proposed to produce a panel of mAbs to heat-killed *L. monocytogenes* and by screening these for serotype and genus specificity to identify listeric antigens which would be useful in serodiagnostic assays.

3.1 Production, identification and characterization of monoclonal antibodies to *Listeria monocytogenes*

3.1.1 Immunization of mice and production of murine hybridomas

Ten female Balb/C mice were immunized with an antigen preparation containing heat-killed *L. monocytogenes* serovar 4b (NCTC 10527). The vaccine was prepared by rehydrating 5 mg dry weight (2.4 mg protein) of bacteria (section 2.7.1) in 10.25 ml of sterile 0.1M Tris-HCl, pH 7.2, 2.25 ml of 2% (w/v) alhydrogel was added and the suspension left at 4°C overnight. For vaccine production the alhydrogel precipitated bacteria in Tris-HCl buffer were emulsified with an equal volume of bayol/arlacel. The final vaccine, containing 200 μg (dry weight) bacteria ml^{-1}, was stored at 4°C. On two occasions four weeks apart mice were injected intraperitoneally with 100 μg (dry weight) antigen (approximately $4x10^8$ c.f.u.) in a 0.5 ml volume of vaccine. Four weeks later the antibody response of each mouse was determined by indirect ELISA (section 2.8.3.1) with heat-killed *L. monocytogenes* serovar 4b (NCTC 10527) as antigen. The mouse with the highest antibody titre was selected and three days prior to fusion boosted by the intravenous injection of 100 μg (dry weight), heat-killed *L. monocytogenes* serovar 4b (NCTC 10527) (section 2.7.1) suspended in PBS. The mouse

was killed, the spleen removed aseptically and the cells fused to cells of the NS-0 plasmacytoma cell line according to the principle described by Kohler and Milstein (1975) using polyethylene glycol as the fusion agent.

3.1.2 Detection of mAbs to *L. monocytogenes*

Twenty two hybridoma cell lines secreting anti-*Listeria* antibodies were detected by indirect ELISA and subsequently all the hybridoma neat supernatant fluids were screened by indirect ELISA (section 2.8.3.1) against both heat-killed *L. monocytogenes* serovar 1/2a (NCTC 7973) and 4b (NCTC 10527) used at concentrations of 80 μg (dry weight) ml^{-1} PBS. All the antibodies recognized both serovars of *Listeria* (Table 3.1) and six of these hybridoma cell lines (25/6, 25/7, 25/10, 25/12, 25/13, 25/25) were selected, stabilized and then cloned by repeated limiting dilution.

Table 3.1.

Detection of *L. monocytogenes* serovars 1/2a and 4b with neat hybridoma cell line supernatant fluid by indirect ELISA

Hybridoma cell line	O.D. at 492nm against: *L. monocytogenes* serovar 1/2a	*L. monocytogenes*. serovar 4b
25/1	0.35	0.23
25/2	0.74	0.60
25/5	1.08	0.77
25/6	**1.44**	**1.11**
25/7	**0.54**	**0.33**
25/8	0.82	0.67
25/10	**1.07**	**0.84**
25/11	1.26	0.85
25/12	**1.43**	**1.17**
25/13	**1.48**	**1.11**
25/14	0.96	0.80
25/15	0.28	0.23
25/16	0.93	0.83
25/17	0.76	0.69
25/18	0.49	0.36
25/19	0.28	0.24
25/21	1.09	0.77
25/22	0.24	0.21
25/23	0.80	0.27
25/24	1.00	0.81
25/25	**1.42**	**1.10**
25/29	0.83	0.69
RPMI+15%FCS	0.00	0.00

(Cell lines shown **emboldened** were selected for further characterization).

3.1.3 Monoclonal antibody isotypes

Using a mouse monoclonal antibody isotyping kit (Amersham International PLC) according to the manufacturer's instructions four of the selected hybridoma cell lines were shown to produce IgG_1 isotype and two IgM isotype antibodies (Table 3.2).

Table 3.2.

Monoclonal antibody isotypes

Hybridoma cell line	Isotype
25/6	IgG_1
25/7	IgM
25/10	IgM
25/12	IgG_1
25/13	IgG_1
25/25	IgG_1

3.2 Comparison of the reactivity of anti-*Listeria* mAbs with *Listeria* species and heterologous bacterial genera

3.2.1 A study of the serovar specificity of the anti-*Listeria* mAbs in dot-blot

Bacteria used were: *L. monocytogenes* serovar 1/2a (NCTC 7973); *L. monocytogenes* serovar 4b (NCTC 10527); *L. ivanovii* serovar 5 (SLCC 2379); *L. innocua* serovar 6a (NCTC 11288). Suspensions of heat-killed organisms were produced as described in section 2.7.1 and each strain was resuspended in PBS at 2 mg (dry weight) ml^{-1} (approximately $8x10^9$ c.f.u. ml^{-1}). 5 μl volumes were dried onto nitrocellulose paper (Schleicher and Schuell) and after blocking free binding sites the sheets were probed with neat anti-*Listeria* mAbs: 25/6, 25/7, 25/10, 25/12, 25/13, 25/25 and anti-*Chlamydia psittaci* mAb 4/17 as negative control (hybridoma cell line supernatant fluids). Rabbit anti-*Listeria* hyperimmune serum (section 2.9.2) diluted 1 in 10 in serum diluent served as positive control. These antisera were incubated at 37°C for 1 h then the assay was continued as described (section 2.8.4).

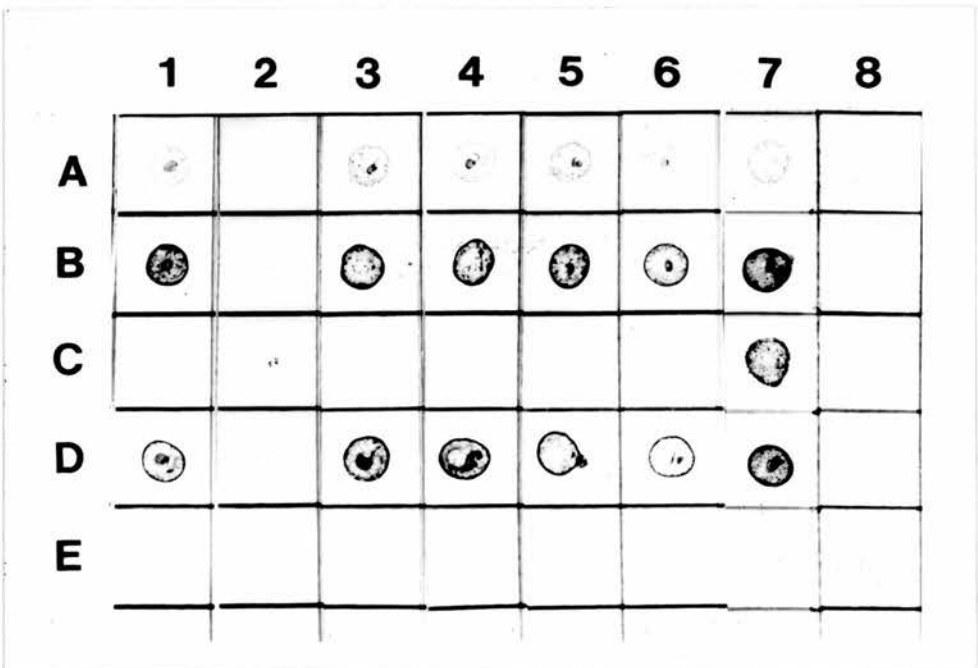
Figure 3.1.

Dot-blot of heat-killed *Listeria* species probed with neat murine monoclonal antibodies and a rabbit anti-*Listeria* hyperimmune serum.

Antigens: row A, *L. monocytogenes* serovar 1/2a; row B, *L. monocytogenes* serovar 4b; row C, *L. ivanovii* serotype 5; row D, *L. innocua* serovar 6a; row E, PBS. 5μl dots of standardized bacterial suspensions dried on nitrocellulose.

Antisera: lane 1, neat culture supernatant fluid of mAb 25/6; lane 2, mAb 25/7; lane 3, mAb 25/10; lane 4, mAb 25/12; lane 5, mAb 25/13; lane 6, mAb 25/25. Lane 7, rabbit anti-*Listeria* hyperimmune serum diluted 1/10 as positive control and lane 8, anti-*Chlamydia psittaci* mAb 4/17 used as negative control.

Figure 3.1.



The anti-*Listeria* mAbs 25/6, 25/10, 25/12, 25/13 and 25/25 all recognized *L. monocytogenes* serovars 1/2a and 4b, together with *L. innocua* serovar 6a (Figure 3.1). The positive control sera recognized *L. monocytogenes* serovars 1/2a, 4b, *L. ivanovii* serotype 5, and *L. innocua* serovar 6a where as no strains were detected by mAb 25/7 or the negative control mAb.

3.2.2 A study of the serovar specificity and recognition of heterologous bacteria by the anti-*Listeria* mAbs in indirect ELISA

To ascertain the specificity of the anti-*Listeria* mAbs they were tested by indirect ELISA against further *Listeria* species and heterologous Gram positive bacteria. Organisms shown in Table 3.3 were grown in TSB to stationary phase at 37°C, collected by centrifugation (4,100 *g* for 30 min at 4°C), washed in PBS and resuspended in 0.5% formol saline for 1 h at 37°C. The final bacterial suspensions were standardized to an O.D. of 0.1 at an absorbance of 420 nm (approximately 10^7 c.f.u. ml^{-1}).

Flat-bottomed microtitre plates (Dynatech M129A) were used and 100 μl of each standardized suspension was added to four wells. Plates were dried overnight and then placed in a microwave cooker (Phillips Cooktronic) for 10 min prior to washing in wash buffer. To each group of four wells neat mAb 25/25 and anti-*Pasteurella multocida* mAb 1/8 (hybridoma cell line supernatant fluids) were added to separate pairs of wells and the assay performed as described (section 2.8.3.1). The optical densities were read at 492 nm and the results are expressed as the mean O.D. with mAb 25/25 - mean O.D. with mAb 1/8 (Table 3.3).

MAb 25/25 detected the following strains: *L. monocytogenes* 1/2a (NCTC 7973), 4b (NCTC 10527), *L. innocua* serovar 6a (NCTC 11288), *L. ivanovii* serotype 5 (SLCC 2379) and isolates belonging to the following *Listeria* serovars: 1/2b, 3b, 4ab, 4b, 4d, also *L. innocua* serovar 6a and *B. subtilis*. There was no recognition of *L. monocytogenes* isolates belonging to serovars: 1/2a, 3c, 4a, 7, *L. ivanovii* serotype 5, *L. grayi*, *L. welshimeri* or the other thirteen heterologous bacteria.

Table 3.3.

Detection of *Listeria* serovars and heterologous species by indirect ELISA and dot-blot

Bacterial species	Source	ELISA O.D. 492nm (mAb 25/25)	Dot-blot (mAbs 25/6, 25/13, and 25/25)
L. monocytogenes serovar 1/2a	(NCTC 7973)	0.25	+/-
L. monocytogenes serovar 4b	(NCTC 10527)	0.27	+
L. ivanovii serovar 5	(SLCC 2379)	0.24	+/-
L. innocua serovar 6a	(NCTC 11288)	0.47	+
L. grayi	a	0.00	-
L. monocytogenes serovar 1/2a	a	0.04	-
L. monocytogenes serovar 1/2b	a	0.58	+
L. monocytogenes serovar 3b	a	0.15	+
L. monocytogenes serovar 3c	a	0.00	-
L. monocytogenes serovar 7	a	0.01	-
L. monocytogenes serovar 4a	a	0.01	-
L. monocytogenes serovar 4ab	a	0.88	+
L. monocytogenes serovar 4b	a	0.57	+
L. monocytogenes serovar 4d	a	1.66	+
L. innocua serovar 6a(4f)	a	0.58	+
L. ivanovii serovar 5	a	0.10	-
L. welshimeri serovar 6	a	0.08	-

Table 3.3. (Cont'd).

Bacterial species	Source	ELISA O.D. 492nm (mAb 25/25)	Dot-blot (mAbs 25/6, 25/13, and 25/25)
Actinomyces pyogenes	b	0.00	-
Bacillus subtilis	c	1.18	+
Erysipelothrix rhusiopathiae	b	0.00	-
Rhodococcus equi	b	0.00	-
Staphylococcus aureus	c	0.01	-
Staphylococcus epidermis	c	0.00	-
Streptococcus agalactiae	b	0.00	-
Streptococcus canis	b	0.00	-
Streptococcus dysgalactiae	b	0.00	-
Streptococcus equi	b	0.00	-
Enterococcus faecalis	b	0.00	-
Enterococcus faecium	d	0.00	-
Streptococcus uberis	b	0.01	-
Streptococcus zooepidemicus	b	0.00	-

Sources: NCTC: National Collection of Type Cultures.
: SLCC: Special *Listeria* Culture Collection.
[a]: Dr. M. Peel. Moredun Research Institute.
[b]: Dr. G. Lawson. R(D)SVS. Edinburgh.
[c]: Dr. W. Donachie. Moredun Research Institute.
[d]: Dr. J. McLauchlin. DMRQC. Colindale.

3.2.3 A study of the serovar specificity and recognition of heterologous bacteria by the anti-*Listeria* mAbs in dot-blot

For the assays 5 µl volumes of the standardized formalin killed suspensions (section 3.2.2) were dried on nitrocellulose paper, free binding sites were blocked, and the sheets incubated in neat hybridoma cell line supernatants of anti-*Listeria* mAbs 25/6, 25/13, 25/25, with anti-*Pasteurella haemolytica* mAb 7/45 as negative control. The assay was continued as described in section 2.8.4.

Except for differences in intensity all three anti-*Listeria* mAbs recognized the same bacterial strains and the results were similar to those obtained by indirect ELISA using mAb 25/25 (Table 3.3).

3.3 Identification and characterization of the antigens recognized by the anti-*Listeria* mAbs

3.3.1 Reactivity of mAbs in immunoblots

Whole cells of *L. monocytogenes* serovar 4b (NCTC 10527) were treated with protease enzymes (section 2.8.6). Together with an untreated control preparation each was diluted 1:1 in double strength SDS-PAGE sample buffer and boiled for 3 min. 25 μl volumes were applied to a SDS-PAGE gel with a 12.5% resolving gel and following electrophoresis were transferred to nitrocellulose paper by immunoblotting (section 2.8.10). After blocking free binding sites strips of the nitrocellulose paper were incubated with either neat hybridoma cell line supernatant fluid of anti-*Listeria* mAb 25/13, anti-*Listeria* flagellin mAb L3/3 or blocking buffer for one hour at 37^{o}C. The assay was then continued as described. MAb 25/13 recognized a heat stable, protease resistant antigen (Figure 3.2; lanes 3, 8, 12) which resolved as a low molecular mass band. Higher molecular mass antigens, in the untreated preparations, reacted nonspecifically with the anti-mouse conjugate and are present in the test and negative control serum lanes (lanes 1, 2, 3, and 4). These bands are lost after protease treatment (lanes 5, 6, 7, 8, 9) and appear to aggregate after boiling (lanes

Figure 3.2.

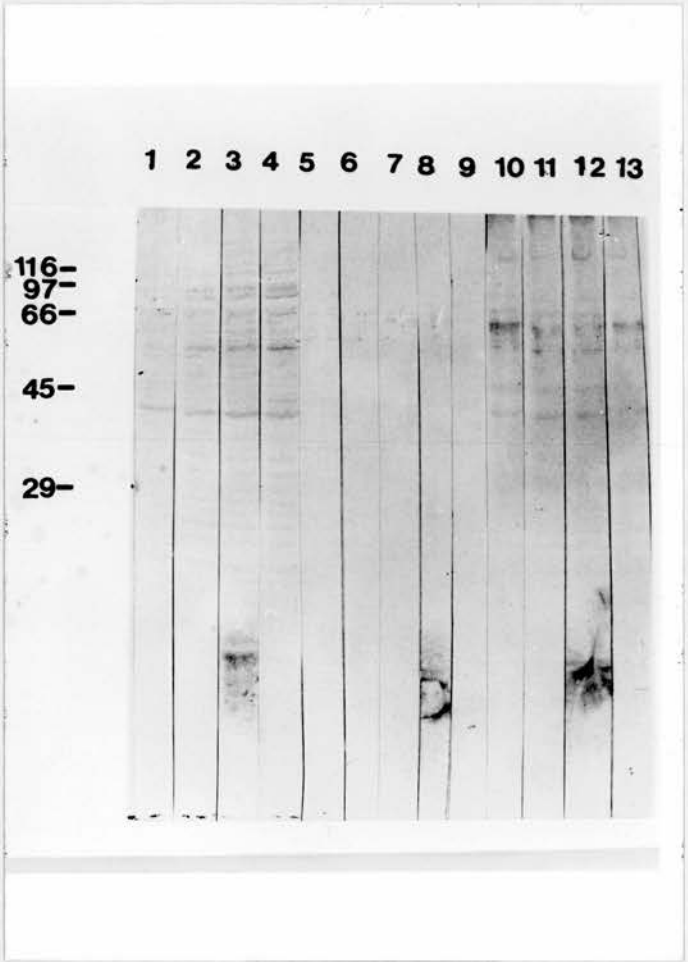
Immunoblot of untreated, boiled and protease treated *in vitro* grown *L. monocytogenes* cells probed with anti-*Listeria* mAb 25/13 and anti-*Listeria* flagellin mAb L3/3.

Antigen: *in vitro* grown *L. monocytogenes* serovar 4b, lanes 1, 2, 3, 4; protease treated *L. monocytogenes* serovar 4b, lanes 5, 6, 7, 8, 9; boiled *L. monocytogenes* serovar 4b, lanes 10, 11, 12, 13.

Antisera: neat culture supernatant fluids of mAb L3/3, lanes 1, 6, 10; mAb 25/13, lanes 3, 8, 12; blocking buffer, lanes 4, 9, 13. Lanes 2, 5, 7, 11 were probed with RPMI+15%FCS.

Position of molecular mass markers (kDa) shown.

Figure 3.2.



10, 11, 12, 13). MAb L3/3 failed to specifically detect any antigen in the preparations.

3.3.2 Hot phenol extraction of antigens recognized by the anti-*L. monocytogenes* mAbs

Hot (80%) phenol extraction was used to produce a preparation, designated PE1, from *L. monocytogenes* serovar 4b (NCTC 10527) using the method described in section 2.7.2. Extracts were prepared for electrophoresis in SDS-PAGE and loaded at 31 μg protein in a single block well of a 16x18 cm x 0.75 mm gel. Following electrophoresis through a 12.5% resolving gel antigens were transferred to nitrocellulose paper as described (section 2.8.10). The nitrocellulose paper was incubated in the neat cell line supernatant fluids of the anti-*Listeria* and anti-*Pasteurella* mAbs listed in Figure 3.3 and the assay continued as before.

Anti-*Listeria* mAbs 25/6, 25/10, 25/12, 25/13, and 25/25 all recognized an antigen present over a considerable area of the nitrocellulose paper in the low molecular mass region with a band of intense staining at approximately 17.5 kDa (marked by an arrow). No antigen was detected by anti-*Listeria* mAb 25/7 or the anti-*Pasteurella* mAbs.

3.3.3 Silver and periodic acid-Schiff staining of phenol extracts in SDS-PAGE gels

A second extraction with hot (80%) phenol (section 2.7.2) produced a preparation referred to as PE2. The extract was resolved with PE1 in SDS-PAGE using the method previously described (section 3.3.2). Antigen loading of PE1 was 0.5 to 2 μg and for PE2, 0.16 to 0.64 μg protein/track. Silver staining, by the method of Tsai and Frasch (1982) (section 2.8.8), revealed bands in PE1 with molecular masses of 19.5, 27 and greater than 45 kDa. A major band at 17.5 kDa was coincidental with a smear in the low molecular mass region (Figure 3.4a). This smeared area was not as obvious using the silver staining method of Morrissey (1981) (section 2.8.8) though the other bands were visible (Figure 3.4b). These bands were not detected in PE2 with either staining method and only the smear in the lower region of the gel was apparent. Periodic acid-Schiff (PAS) staining was carried out according to the method of Zacharius *et al.* (1969) (section 2.8.9) and the low molecular mass smear visualized in both PE1 and PE2 extracts (photographic representation of this is too poor for reproduction).

Figure 3.4.

Silver staining of phenol extracts PE1 and PE2 in SDS-PAGE.

a. Silver stain (Tsai and Frasch, 1982). Antigen: lane 1, PE1 (0.5 μg protein); lane 2, PE1 (1 μg protein); lane 3, PE1 (1.5 μg protein); lane 4, PE1 (2.0 μg protein); lane 5, PE2 (0.16 μg protein); lane 6, PE2 (0.32 μg protein); lane 7, PE2 (0.48 μg protein); lane 8, PE2 (0.64 μg protein).

b. Silver stain (Morrissey, 1981).
Antigen: lane 1, PE1 (1 μg protein); lane 2, PE2 (0.32 μg protein); lane 3, PE1 (1.5 μg protein); lane 4, PE2 (0.48 μg protein).

Position of molecular mass markers (kDa) shown.

Figure 3.4a.

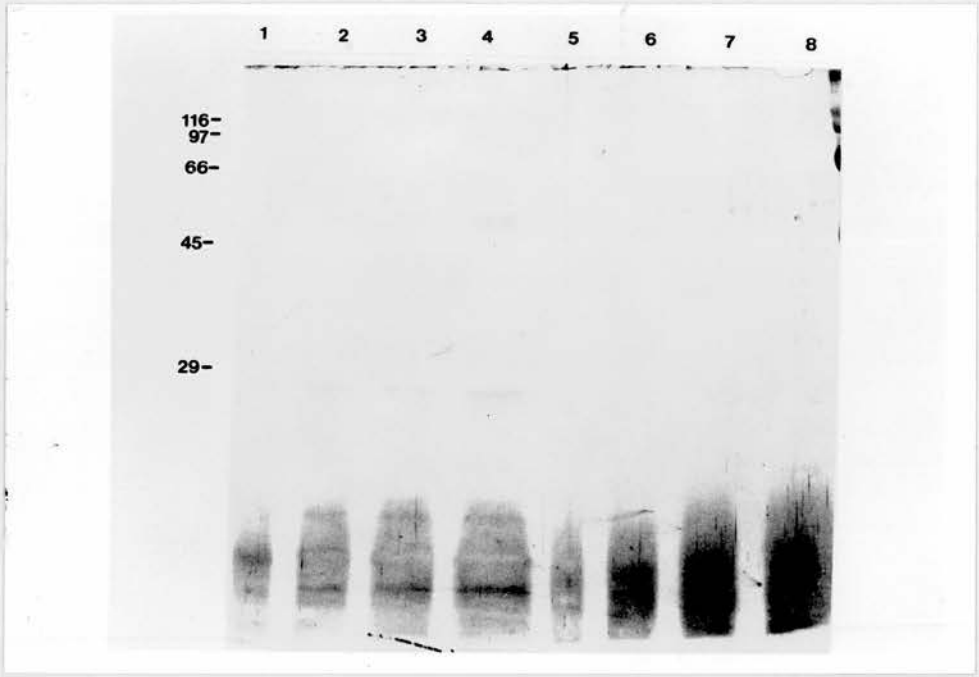
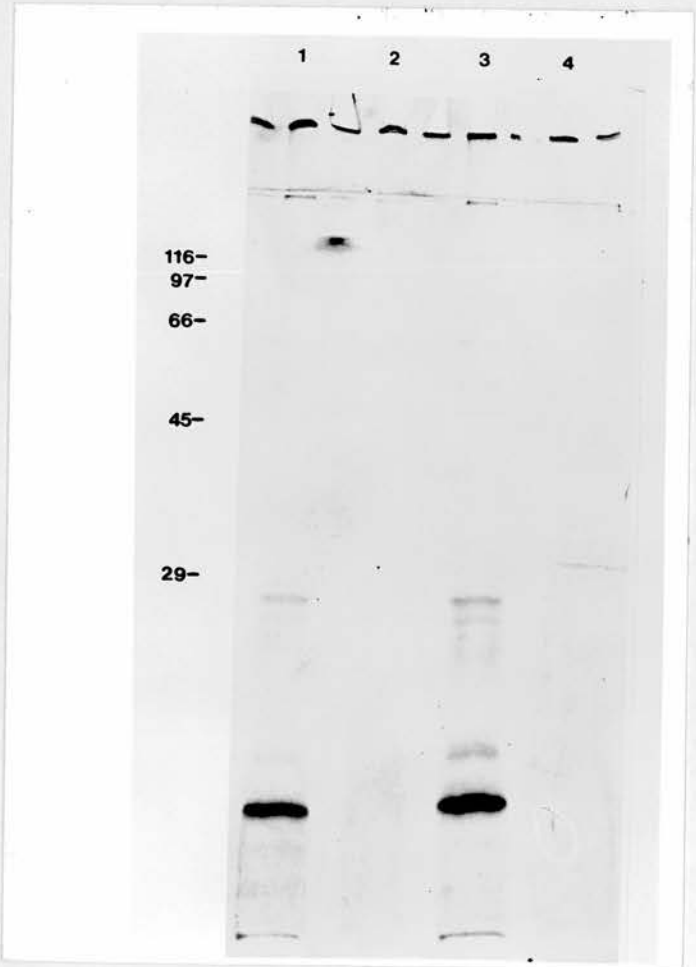


Figure 3.4b.



3.3.4 Protein and carbohydrate analysis of PE1 and PE2

Protein analyses were performed with the BCA enhanced protein assay (section 2.8.13). PE1 contained 125 μg, and PE2: 32.5 μg protein ml^{-1}. Using the method of Dubois *et al.* (1956) PE1 was shown to contain 315 μg and PE2: 63 μg carbohydrate ml^{-1}.

3.3.5 Passive haemagglutination of PE2 coated erythrocytes by anti-*Listeria* mAb 25/10

Passive haemagglutination assays were carried out as described in section 2.8.11. 50 μl of RPMI+15%FCS and hybridoma cell line supernatant fluids of anti-*Listeria* flagellin mAb L3/3, anti-*Listeria* mAbs 25/10 and 25/25 were double diluted from neat to a dilution of 1/2048 in PBS in U bottomed microtitre plates. 50 μl volumes of untreated erythrocytes and 50 μl of each preparation of PE2 pretreated erythrocytes were added to each antibody dilution. Plates were thoroughly mixed by shaking and left at room temperature overnight. Results were expressed as the reciprocal of the final dilution at which haemagglutination occurred.

MAb 25/10 produced a titre of 16 with erythrocytes pretreated in 1/10 PE2, and 8 with 1/100 PE2 treated erythrocytes. A marked prozone effect occurred with erythrocytes pretreated in 1/1000 PE2 and haemagglutination was only clearly visible at the 1/8 antiserum dilution. No haemagglutination occurred with untreated erythrocytes in mAb 25/10 or with any of the erythrocyte preparations in: RPMI+15%FCS, mAbs L3/3 or 25/25 (Figure 3.5).

Figure 3.5.

Agglutination of PE2 coated sheep red blood cells by anti-*Listeria* mAb 25/10.

a. Rows A to D, RPMI+15%FCS double diluted in PBS; rows E to H, neat anti-*Listeria* flagellin mAb L3/3 double diluted in PBS in lanes 1-12. 50 μl of a 1% concentration of uncoated erythrocytes added to each well of rows A and E. 1/10 PE2 coated erythrocytes added to each well of rows B and F. 1/100 PE2 coated erythrocytes added to each well in rows C and G. Rows D and H, 1/1000 PE2 coated erythrocytes added to each well.

b. Rows A to D, neat anti-*Listeria* mAb 25/25 double diluted in PBS; rows E to H, neat anti-*Listeria* mAb 25/10 double diluted in PBS in lanes 1-12. 50 μl of a 1% concentration of uncoated erythrocytes added to each well of rows A and E. 1/10 PE2 coated erythrocytes added to each well of rows B and F. 1/100 PE2 coated erythrocytes added to each well in rows C and G. Rows D and H, 1/1000 PE2 coated erythrocytes added to each well.

Figure 3.5a.

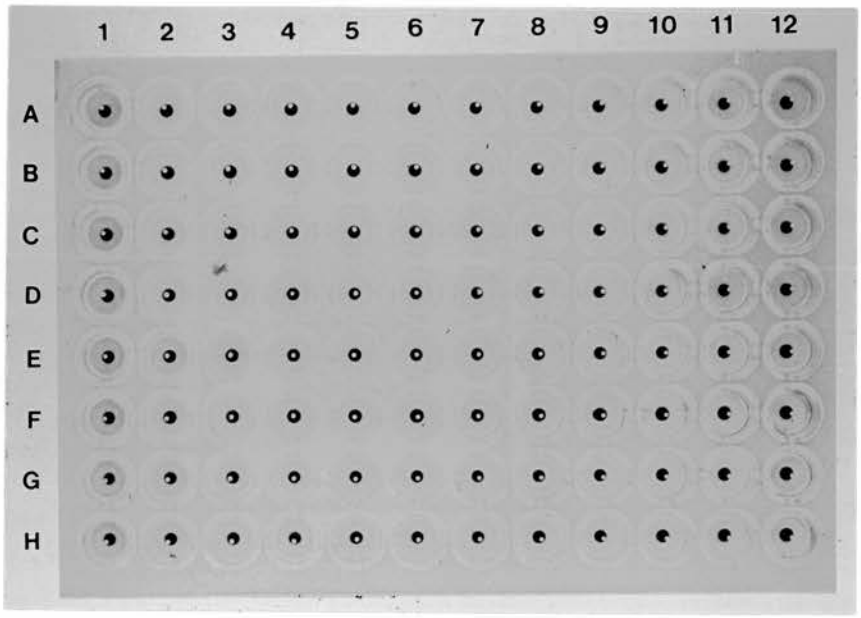
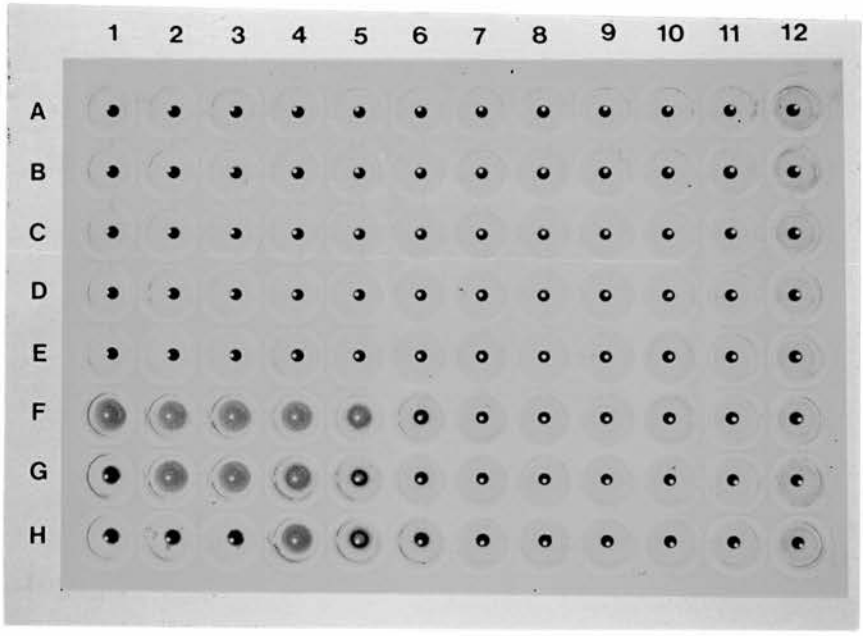


Figure 3.5b.



3.4 Development of an antigen sandwich ELISA and an examination of the anti-*Listeria* mAbs for competition in antigen binding

3.4.1 Production of ascites in mice

Adult Balb/c female mice were used for the production of ascitic fluid by the hybridoma cell lines (section 2.9.1).

3.4.2 Purification of antibody from mouse ascitic fluid

Antibody was purified from ascitic fluid produced by hybridoma cell lines 25/12, 25/13 and 25/25 using caprylic acid (section 2.9.1). Protein concentrations were estimated from the absorbance at 280 nm (section 2.8.13) and each mAb was standardized in sterile PBS to 500 μg protein ml^{-1}. Purification of antibody from ascitic fluid produced by hybridoma cell line 25/25 was also carried out by affinity chromatography on protein G-Sepharose and the method of Wilson and Nakane (1978) was used to conjugate the purified mAb to horseradish peroxidase.

3.4.3 Development of an antigen sandwich ELISA using the purified mAbs

Purified mAbs of cell lines 25/25, 25/12, 25/13 (ascitic fluids) were diluted to 50 μg ml^{-1} in sterile PBS and 100 μl added to wells of 96 well flat-bottomed microtitre plates. Plates were left overnight at 4°C before washing and addition of 4% (w/v) skim milk powder in PBS to all wells. After incubation for 1 h followed by thorough washing PE2, double diluted from 1/1600 to 1/51200 in serum buffer, was added to six rows on each plate in 100 μl volumes. Negative control wells contained 100 μl serum buffer alone. Plates were incubated for 90 min at 37°C and after thorough washing horseradish peroxidase conjugated mAb 25/25, double diluted from 1/200 to 1/1600 in serum buffer, was added as 100 μl per well in a chequer board pattern. Plates were incubated at 37°C for 90 min and the assay continued as described in section 2.8.3.1. Results were expressed as the mean O.D. 492 nm - mean O.D. 492 nm without antigen (Figures 3.6, 3.7, 3.8). The homologous system using mAb 25/25 as the capture antibody was as effective as the heterologous systems with either mAb 25/12 or 25/13. In all the assays the increasing dilution of PE2 lead to a reduction in the absorbance values and a linear titration of log conjugate dilution against absorbance was demonstated.

Figure 3.6.

Capture of PE2 (diluted from 1/1600 to 1/51200 in serum buffer) in sandwich ELISA using purified anti-*Listeria* mAb 25/25 (5 μg per well) with detection by HRP conjugated mAb 25/25 (diluted from 1/200 to 1/1600).

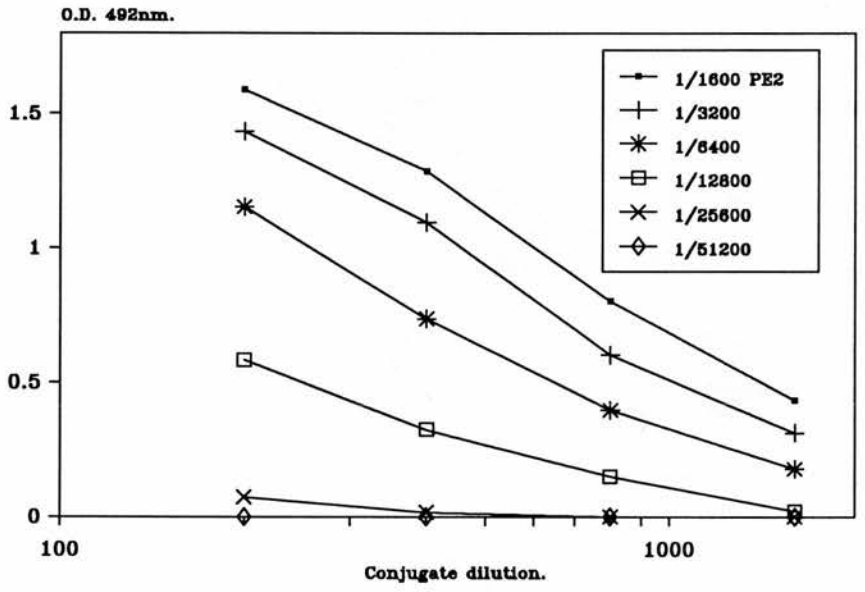


Figure 3.7.

Capture of PE2 (diluted from 1/1600 to 1/51200 in serum buffer) in sandwich ELISA using purified anti-*Listeria* mAb 25/12 (5 μg per well) with detection by HRP conjugated mAb 25/25 (diluted from 1/200 to 1/1600).

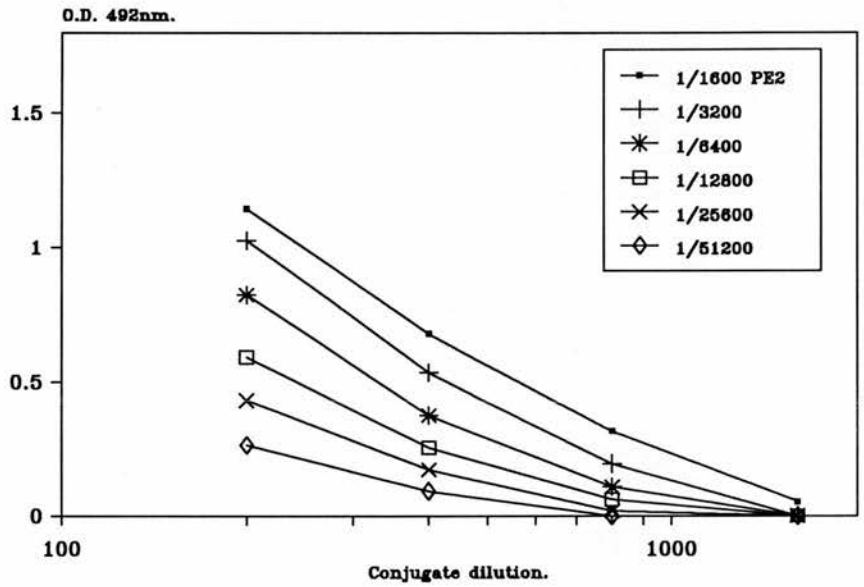
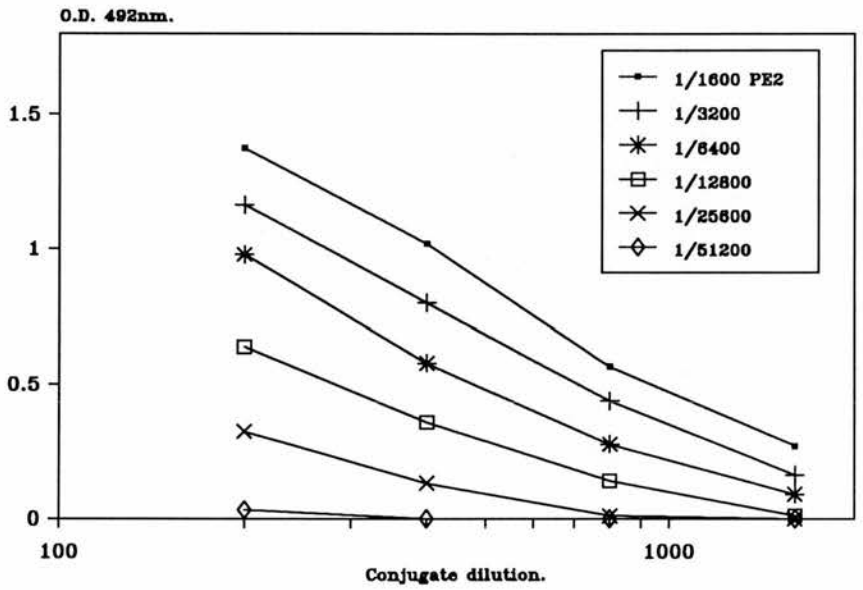


Figure 3.8.

Capture of PE2 (diluted from 1/1600 to 1/51200 in serum buffer) in sandwich ELISA using purified anti-*Listeria* mAb 25/13 (5 μg per well) with detection by HRP conjugated mAb 25/25 (diluted from 1/200 to 1/1600).

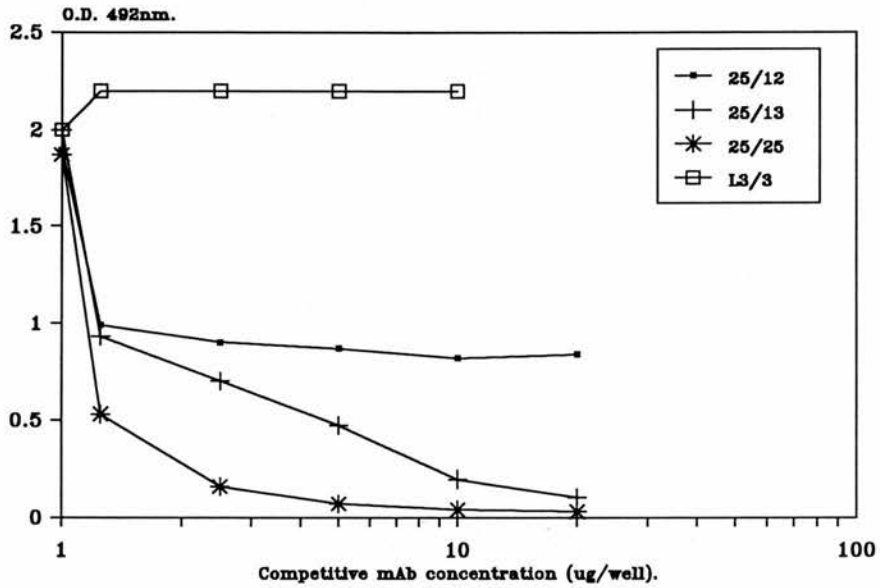


3.4.4 Competition for epitope binding by the anti-*Listeria* mAbs 25/12, 25/13, 25/25 in a sandwich ELISA

Purified mAb 25/25 (ascitic fluid) was diluted to 50 μg ml^{-1} in sterile PBS and 100 μl added to the wells of a 96 well flat-bottomed microtitre plate. Plates were left overnight at 4°C before washing and addition of 4% (w/v) skim milk powder in PBS to all wells. After incubation, then thorough washing, excess antigen in the form of 100 μl of a 1/800 dilution of PE2 in serum buffer was added to all wells and plates were incubated for 90 min at 37°C. Purified ascites from mAbs 25/12, 25/13 and 25/25 standardized to 200 μg ml^{-1} were double diluted in serum buffer and with neat ascitic fluid of anti-*Listeria* flagellin mAb L3/3 double diluted to 1/16 added as 100 μl volumes to wells in separate rows. After incubation at 37°C for 90 min and thorough washing HRP conjugated mAb 25/25 at a 1/400 dilution in serum buffer was added to all wells and incubated at 37°C for 90 min. The assay was continued as described in section 2.8.3.1. Optical densities were read at 492 nm and the results are shown in Figure 3.9. The reduction in the absorbances revealed that all three anti-*Listeria* mAbs 25/12, 25/13, and 25/25 competed with HRP conjugated 25/25. The negative control anti-*Listeria* flagellin mAb L3/3 produced no reduction in the absorbance at 492 nm.

Figure 3.9.

Competition for epitope binding by the anti-*Listeria* mAbs 25/12, 25/13, 25/25 in a sandwich ELISA.



The standard was determined using 5 μg purified 25/25 mAb per well as capture antibody, with a 1/800 dilution of PE2 as antigen and a 1/400 dilution of HRP conjugated mAb 25/25 as detecting antibody. Competition was determined through the reduction in optical density with purified mAbs 25/12, 25/13 and 25/25. Negative control was anti-*Listeria* flagellin mAb L3/3 (ascitic fluid diluted from 1/2 to 1/16).

Discussion

Heat-killed bacteria are recommended for the production of antibodies to *Listeria* somatic antigens (Seeliger and Hohne, 1979) and twenty two hybridoma cell lines which secreted anti-*Listeria* antibodies were successfully produced following the immunization of mice with heat-killed *L. monocytogenes* serovar 4b. To distinguish serotype specific mAbs neat culture supernatant fluids were tested by indirect ELISA against heat-killed *L. monocytogenes* serovars 1/2a and 4b. However, all the mAbs detected both strains which share no somatic antigens other than the common antigen 3 (Seeliger and Jones, 1986).

Five of the six anti-*Listeria* mAbs chosen for characterization identified an antigen common to heat-killed *L. monocytogenes* serovars 1/2a, 4b, *L. innocua* serovar 6a but not *L. ivanovii* serotype 5, though the last strain inactivated in formol saline was recognized in both dot-blot and indirect ELISA. This inconsistency may be explained by the recognized interference which can occur in the antibody antigen reaction through the masking of epitopes by the aggregation of heat denatured surface proteins (Robbins and Griffin, 1945; Osebold *et al.*, 1965). The results were inconsistent with the detection of serotype specific antigens and the

immunization protocol was thus unsuccessful for the production of a battery of mAbs to listeric somatic antigens as defined by the Seeliger/Donker-Voet classification system (Seeliger and Jones, 1986).

A study of the mAbs reactivity with seventeen *Listeria* strains and heterologous bacteria showed that five of the six recognized an antigen irregularly present in a number of species and serovars of the genus *Listeria*. The major somatic factors 1, 2, 4 and 5 are known to be heat stable (Robbins and Griffin, 1945, Seeliger and Hohne, 1979) and are possibly carbohydrate in nature (Osebold *et al.*, 1965; Ullmann and Cameron, 1969). The character of the minor somatic factor 3 is equivocal (Robbins and Griffin, 1945) and its presence irregular (Gray and Killinger, 1966). Since the mAbs detected a heat stable antigen occasionally present on a number of *Listeria* serotypes the identified antigen was possibly the common somatic antigen 3 and therefore relevant to the serological classification of *Listeria* species. Though the mAbs also recognized *B. subtilis* they were used for the development of an assay to detect anti-*Listeria* antibodies. Further characterization of the mAbs and an attempt at identification of the recognized antigen were also undertaken.

MAb 25/7 failed to detect any of the heat-killed *Listeria* species in dot-blot and did not detect any antigen in immunoblots of the phenol extracts. Its reactivity in the initial indirect ELISA is inexplicable.

In immunoblots mAb 25/13 recognized a heat stable antigen which was resistant to protease digestion and appeared as a smear in the low molecular mass region in immunoblots. A consideration of the cell wall structure of *Listeria* species (Fiedler *et al.*, 1984; Fiedler and Ruhland, 1987) led to the conclusion that the antigen was either cell wall teichoic acid or cytoplasmic membrane lipoteichoic acid (LTA). Its appearance in immunoblots was reminiscent of the appearance of Gram negative rough form LPS (Pyle and Schill, 1985) and of the atypical cell wall carbohydrate of *Clostridium difficile* (Sharp and Poxton, 1986) and the antigen was putatively identified as LTA.

Hot phenol can be used to extract LTA from a variety of Gram positive bacterial cells, including *L. monocytogenes* (Wicken and Knox, 1975; Fischer *et al.*, 1983; Hether and Jackson, 1983; Uchikawa *et al.*, 1986b) and the putative identity of the antigen under investigation was partly confirmed by the successful phenol extraction of an antigen with which five of the six mAbs reacted. The antigen also spontaneously adhered to sheep erythrocytes, as is typical for LTA (Knox and Wicken, 1973; Wicken and

Knox, 1975; Hether and Jackson, 1983) and could be detected in passive haemagglutination tests using the IgM isotype mAb 25/10. That the antigen coated erythrocytes could not be agglutinated by the IgG_1 isotype mAb 25/25 is attributed to the inability of these antibodies to initiate agglutination.

To further elucidate the nature of the antigen the compositions of the phenol extracts PE1 and PE2 were examined in basic biochemical tests. Crude phenol extracts often suffer from substantial contamination of the LTA by proteins, polysaccharides, teichoic acids and nuclease resistant nucleic acids (Fischer *et al.*, 1983) and the considerable variation between the carbohydrate and protein contents of PE1 and PE2 are assumed to be due to the presence of such impurities.

Additional examinations were carried out by staining the antigen in SDS-PAGE gels and the results revealed long smears in the low molecular mass region in both PE1 and PE2 identical to the antigen detected in immunoblots by the anti-*Listeria* mAbs. Following periodate oxidation this antigen could be stained using the silver staining method of Tsai and Frasch (1982) and less intensely with fuchsin by the PAS method of Zacharius *et al.* (1969). Silver staining using the method of Morrissey (1981), in which no periodate oxidation is involved, was successful

though the staining was less intense. These results suggested the presence of hydroxyl groups on adjacent carbon atoms which could be cleaved by periodate oxidation yielding two terminal aldehyde groups able to reduce silver nitrate to metallic silver or to form covalent bonds with fuschin sulphite. Other bands in PE1, absent or diminished in PE2, were nonreactive in immunoblots and were assumed to be contaminant proteins.

The published structure of the LTA molecule (Appendix 1) reveals a number of potential periodate cleavage sites both within the glycosyl side chain substituents and in the linkage disaccharide between the terminal phosphomonoester of the polyglycerol phosphate chain and the hydrophobic acyl groups (Uchikawa *et al.*, 1986b; Ruhland and Fiedler, 1987). Since the mean chain length of LTA molecules varies considerably (Uchikawa *et al.*, 1986b) the smearing of the stains in SDS-PAGE and of the antigen in immunoblots may be attributed to variation in molecular size and can be taken as additional evidence of the nature of the antigen.

Subsequent examination of the epitope recognized by three of the mAbs was achieved through the development of a sandwich ELISA. Antigen capture and detection was acheived with a single mAb and indicated the presence of multiple antigenic determinants per molecule as is typical of many carbohydrate antigens (Goding, 1986). Since two of the IgG_1 isotype mAbs competed with mAb 25/25 in a competitive sandwich ELISA these mAbs presumably recognise the same or overlapping epitopes. A modification of this assay, described in section 2.8.3.2, was developed to measure anti-LTA antibody levels in sheep serum and an examination is made in Chapter 6 of the detection of anti-LTA antibody responses in experimentally infected animals.

Chapter 4.

Purification of listeriolysin O and development of an immunoassay for the detection of anti-listeriolysin O antibodies in sheep.

Introduction

Listeriolysin O (LLO) is a major virulence factor produced by all pathogenic strains of *Listeria monocytogenes* and is antigenically related to other sulphydryl-activated toxins produced by members of the genera *Streptococcus, Bacillus, Clostridium* and *Listeria* (Alouf and Geoffroy, 1984; Geoffroy *et al.*, 1987). However, Geoffroy and Alouf (1984) described antigenic variation in these toxins and the gene sequencing studies of Mengaud *et al.* (1987) have also revealed structural differences between streptolysin O (SLO), LLO and pneumolysin. It is thus feasible that an immunoassay based upon purified LLO can be used as an indicator of listeric infections in sheep. The purification of LLO, development of an indirect ELISA based upon the antigen and production of a hyperimmune anti-LLO serum are described.

4.1 Production of haemolysin by *Listeria monocytogenes* serovar 4b (strain L1059)

4.1.1 Determination of haemolysin production in broth culture

A wild strain of *L. monocytogenes* serovar 4b (L1059) was used throughout this chapter. The organism was maintained in PBS at -70°C. Prior to growth in broth cultures it was grown overnight at 37°C on 5% sheep blood agar plates. Two 400 ml volumes of BHIB (Gibco) were inoculated with 4 ml BHIB containing approximately $5x10^9$ c.f.u. *L. monocytogenes* at stationary phase. The bottles were incubated, without shaking at 37°C, and paired 1 ml samples were taken at 2 h intervals over the following 24 h. The optical density at 600 nm was determined and the haemolytic activity, following filtration through a 0.45 μm membrane filter (Millipore), was measured according to the method in section 2.8.12. Haemolysin production peaked after the logarithmic phase of bacterial growth and haemolytic activity declined considerably from 18 to 24 h (Table 4.1).

Table 4.1.

Haemolysin production by *L. monocytogenes* (L1059) in broth culture

Time (hours)	Optical density (A_{600} nm)	Haemolytic activity (HU).
0	0.00	0
2	0.09	1
4	0.60	1
6	0.92	32
8	0.92	128
10	0.92	128
12	0.90	128
18	0.81	128
24	0.62	32

4.1.2 Determination of haemolysin production in dialysis sac broth cultures

A culture of *L. monocytogenes* was grown in 10 ml of BHIB (Gibco) for 4 h at 37°C. The broth was centrifuged (2,000 *g* for 15 min at 18°C) and the bacterial pellet resuspended in 100 ml of sterile PBS. Taking aseptic precautions and using sterile materials and equipment 10 ml aliquots were dispensed into ten individual lengths of dialysis membrane (Visking, TWT-400-070M) 31 mm width. These were suspended in ten separate 500 ml volumes of BHIB (Gibco) and incubated at 37°C without shaking. Paired 1 ml samples were taken from the dialysis sac cultures at two hour intervals over the following 12 h and the optical density at 600 nm and haemolytic activity were measured as previously described. Though haemolytic titres were lower than in broth cultures the peak of haemolysin production again occurred following the logarithmic phase of growth (Table 4.2).

Table 4.2.

Haemolysin production by *L. monocytogenes* (L1059) in dialysis sac broth cultures

Time (hours)	Optical density (A_{600} nm)	Haemolytic activity (HU).
0	0.56	0
4	1.18	0
6	1.54	1
8	1.50	16
10	1.50	64
12	1.65	64

4.2 Purification of LLO

4.2.1 Bacterial culture

An overnight growth of *L. monocytogenes* serovar 4b was obtained by culture on 5% sheep blood agar plates. The organism was grown thereafter in 10 ml of BHIB (Gibco) for 4 h at 37°C. The broth was centrifuged (2,000 *g* for 15 min at 18°C) and the bacterial pellet (approx. $1x10^7$ c.f.u. ml^{-1}) resuspended in 100 ml of sterile PBS. Taking aseptic precautions and using sterile materials and equipment the suspension was poured through a funnel into a two metre length of dialysis membrane (Visking, TWT-400-070M), 31mm width, sealed at one end and suspended in 5 l of BHIB (Gibco). With the upper end trapped by the bottle cap, the culture was incubated at 37°C for 12 h without stirring.

4.2.2 Harvesting of supernatant fluid

The contents of the dialysis sac were collected and centrifuged (4,300 *g* for 45 min at 4°C). The purity and identity of the organism growing within the dialysis sac were checked by bacterial culture. The supernatant fluid was filtered through a 0.45 μm filter membrane (Millipore) and dialysed at 4°C against 5mM 2-[N-morpholino]ethanesulphonic acid (MES, Sigma) adjusted to pH 6.5 with NaOH. The 1 l dialysis buffer volume was changed six times over 24 h. The dialysed supernatant fluid (approximately 80 ml) was lyophilized (Edwards, Super Modulyo freeze dryer) and typically produced 280 mg freeze dried material from each dialysis sac.

4.2.3 Purification of listeriolysin O by SP-cation exchange chromatography

Lyophilized supernatant fluid was rehydrated by addition of one tenth the original volume of distilled water containing 5% (v/v) glycerol and 1mM ß-mercaptoethanol (Sigma). A 8x75 mm Glaspac TSK-SP-5PW high performance liquid chromatography column (Pharmacia LKB) was equilibrated at room temperature with 50mM MES, 1mM ß-mercaptoethanol, 5% (v/v) glycerol, pH 6.5 buffer. The concentrated supernatant fluid was applied to the column by the multiple application of 2 ml volumes and the

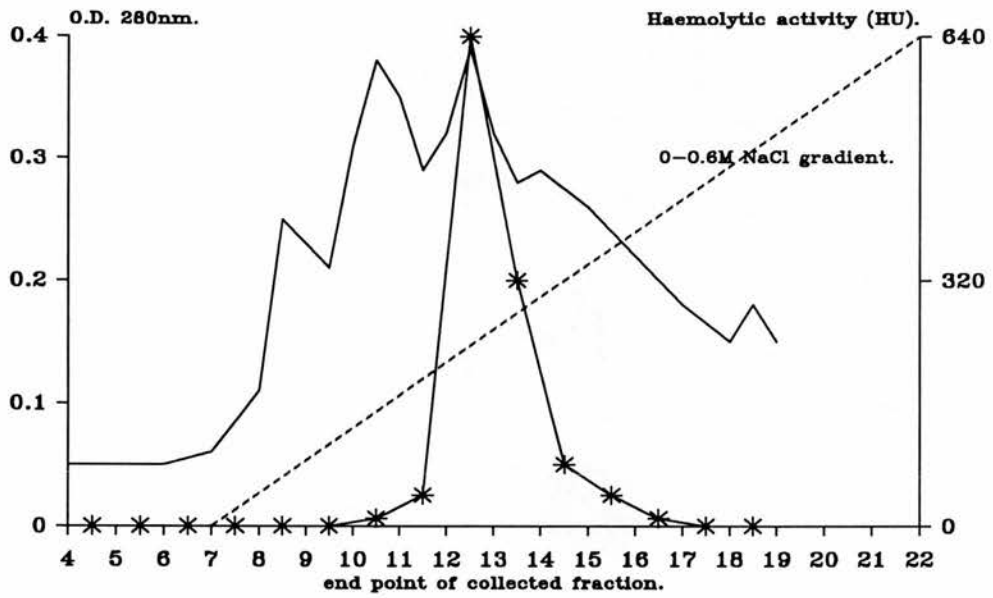
column was washed exhaustively with starting buffer. The haemolysin was eluted with a 0-0.6M NaCl gradient applied over 30 min at a flow rate of 0.5 ml min^{-1}. Fractions were collected every 2 min.

The absorbance at 280 nm and the haemolytic activity of column fractions were monitored. The purity of LLO was assessed in SDS-PAGE by silver staining (Morrissey, 1981) and immunoblotting (section 2.8.10). Fractions containing peak haemolytic activity were stored at -70°C.

Chromatography of the concentrated supernatant fluid on the SP-cation exchange column produced two major peaks of absorbance. Haemolysin eluted reproducibly from the column at 210-280mM NaCl, pH 6.5, as the second major peak and was collected in fraction 13 (Figure 4.1). This fraction retained a haemolytic activity of 640 HU and contained approximately 50 μg protein ml^{-1}, representing approximately 20 per cent recovery of lytic activity and a 120 fold purification (Table 4.3). In SDS-PAGE fraction 13 was shown by silver staining to contain a single homogeneous protein with a molecular mass of 58 kDa (Figure 4.2). The protein was recognised in immunoblots both by the goat convalescent serum (section 2.9.3) as shown in figure 4.3 and by a hyperimmune anti-LLO rabbit serum (section 2.9.4) as shown in figure 4.8.

Figure 4.1.

SP-cation exchange fractionation of *L. monocytogenes* dialysis sac culture supernatant fluid.



—— Haemolytic activity (HU).

- - - 0 - 0.6M NaCl gradient.

——— O.D. 280 nm.

Figure 4.2.

Silver staining of dialysis sac culture supernatant fluid and SP-cation exchange fractions in SDS-PAGE.

Lane 1, *L. monocytogenes* dialysis sac supernatant fluid post dialysis (4 μg protein); lane 2, 10X dialysis sac supernatant fluid (32.5 μg protein); lane 3, SP-cation exchange column fall through (15 μg protein); lane 4, fraction 10 collected from SP-cation exchange column (0.5 μg protein); lane 5, fraction 12 (0.1 μg protein); lane 6, fraction 13 (0.5 μg protein); lane 7, fraction 14 (0.4 μg protein); lane 8, fraction 16 (0.2 μg protein).

Position of molecular mass markers (kDa) shown.

Figure 4.2.

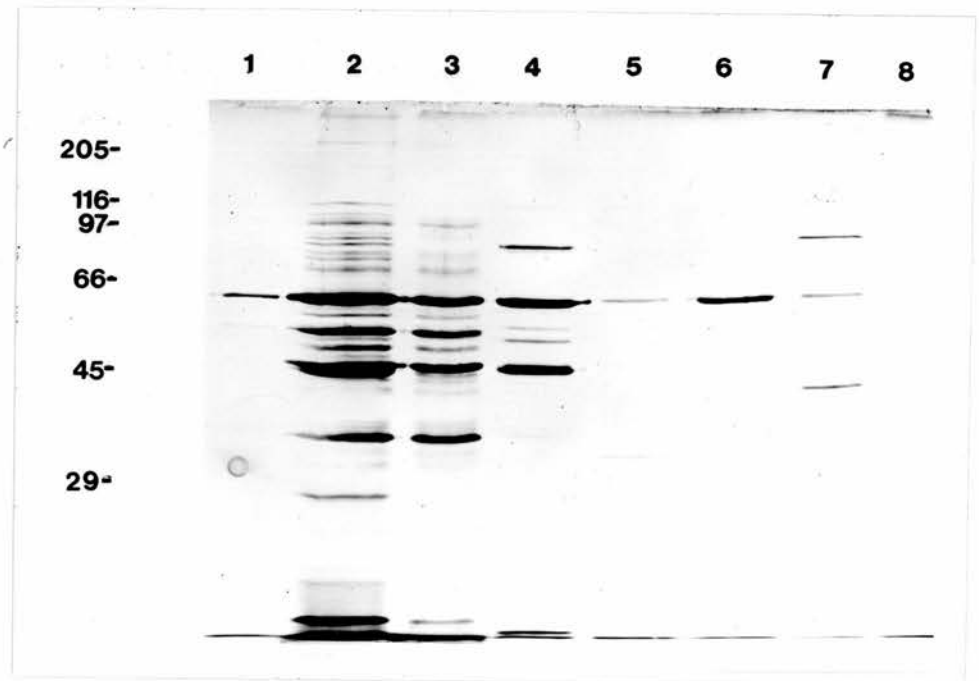


Figure 4.3.

Immunoblot of dialysis sac culture supernatant fluid and SP-cation exchange fractions using goat convalescent serum.

Lane 1, *L. monocytogenes* dialysis sac supernatant fluid post dialysis (4 μg protein); lane 2, 10X dialysis sac supernatant fluid (32.5 μg protein); lane 3, SP-cation exchange column fall through (15 μg protein); lane 4, fraction 10 collected from SP-cation exchange column (0.5 μg protein); lane 5, fraction 12 (0.1 μg protein); lane 6, fraction 13 (0.5 μg protein); lane 7, fraction 14 (0.4 μg protein); lane 8, fraction 16 (0.2 μg protein).

Goat convalescent serum used at a dilution of 1/500.

Position of molecular mass markers (kDa) shown.

Figure 4.3.

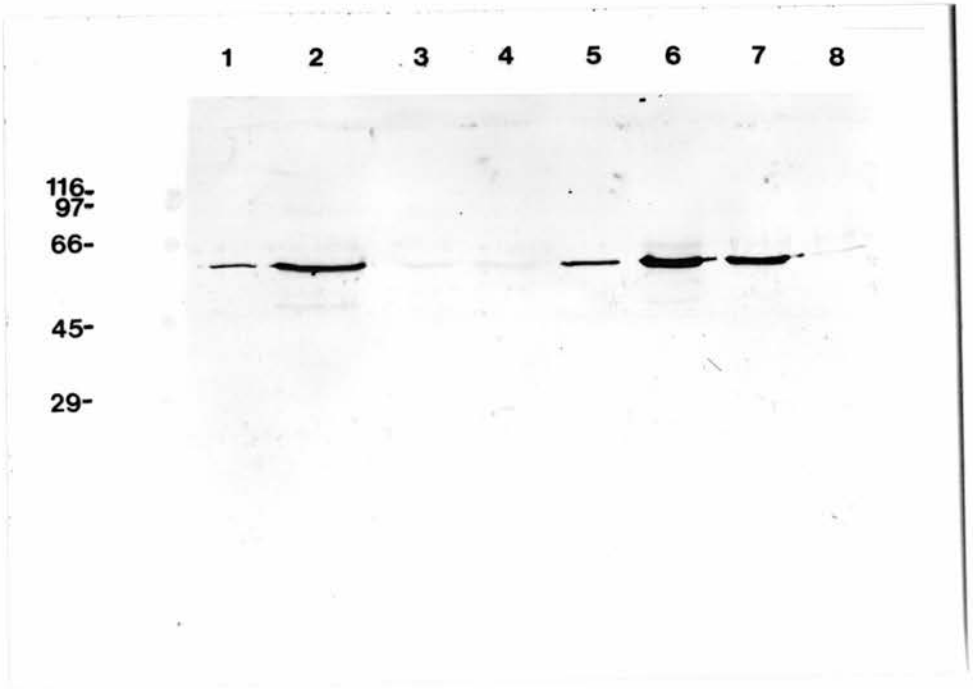


Table 4.3.

Purification of LLO

Purification step	Total protein (mg)	Total haemolytic activity (HU)	Specific activity (HU/mg protein)	Recovery (%)
1. Culture supernatant fluid (80 ml).	30.0	3,200	106.7	100
2. Conc'd supernatant fluid (8.8 ml).	27.3	2,688	98.5	84
3. Chromatography peak fraction (1 ml).	0.05	640	12,800	20

4.3 Development of an indirect ELISA using purified LLO as antigen

4.3.1 Experimental sera

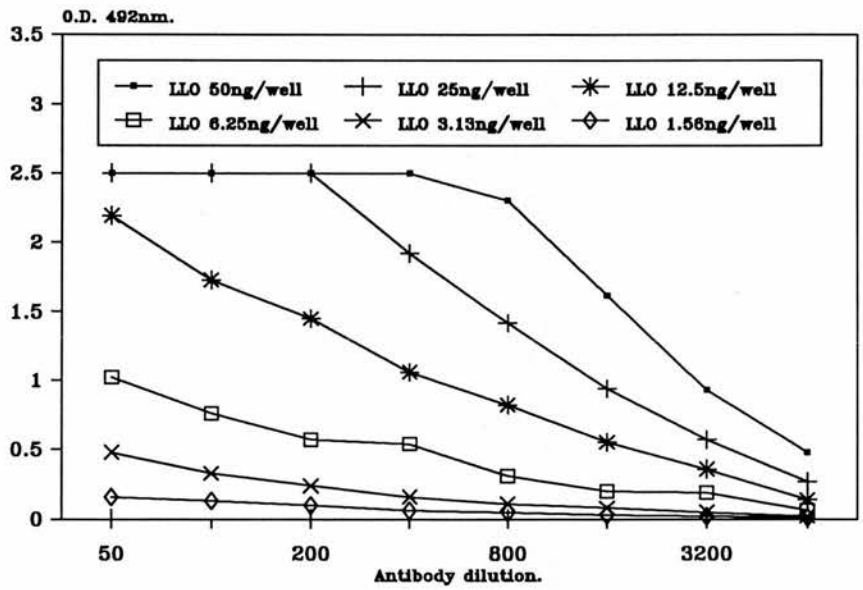
Details of the experimental challenge of conventional lambs with *L. monocytogenes* are given in Chapter 5 and sera derived from these experiments were used in the development of the anti-LLO immunoassay. Briefly, the titration of antibody against antigen was performed with: serum 1 collected before experimental challenge; serum 2 collected three weeks after oral dosing with *L. monocytogenes*; and sera 3 and 4 taken from two lambs 28 days after subcutaneous injection with the organism.

4.3.3 Determination of the optimal antigen dilution in indirect ELISA

Sterile 96 well microtitre plates (Dynatech M129A) were used throughout. A chequer board titration was performed with serial dilutions of purified LLO (chromatography fraction 13) in 0.1M carbonate/bicarbonate buffer, pH 9.6, against doubling dilutions of the goat convalescent serum (section 2.9.3). Plates were coated with antigen dilutions (100 μl per well) and left sealed at 4^{o}C overnight. After repeated washing with wash buffer, 100 μl of blocking buffer, 5% (w/v) skim milk powder in PBS, was added to all wells and the plates left sealed at 37^{o}C for 1 h. After washing, serum dilutions in serum buffer were added in 100 μl volumes and plates again incubated sealed at 37^{o}C for 1 h. Plates were washed as before and 100 μl donkey anti-sheep HRP conjugate (section 2.8.1) diluted 1/200 in serum buffer was added to all wells. Sealed plates were again incubated for 60 min at 37^{o}C before washing and continuation of the assay as described in section 2.8.3.1. The log dose response curve was linear using antigen coating of 6.25 and 12.5 ng per well. At 25 ng per well a plateau effect was seen with serum dilutions between 1/50 and 1/200 (Figure 4.4).

Figure 4.4.

Titration of convalescent serum (diluted from 1/50 to 1/6400) against purified LLO (at concentrations of 1.56 ng per well to 50 ng per well) in indirect ELISA.



4.3.4 Titration of positive and negative experimental sera against purified LLO in indirect ELISA

Sera collected from conventional animals before and after experimental challenge with *L. monocytogenes* were titrated against purified LLO at dilutions of 6.25, 12.5 and 25 ng per well. Plates were coated and assays performed as described (section 4.3.3). The results of titration of these known experimental sera against antigen are shown in Figures 4.5, 4.6 and 4.7. The results for the experimental serum 1 are not shown as the optical density at 492 nm was less than 0.05 for all antibody dilutions at all antigen concentrations. With antigen coating of 25 ng per well the three positive control sera produced optical density results in the range of 0.2 to 1.4 at serum dilutions of 1/200 to 1/800, and the log dose response curve was linear for each antibody tested. For each serum, increasing antibody dilution was shown to have less effect on optical density than decreasing the antigen coating.

Figure 4.5.

Titration of experimental serum 2 (diluted from 1/200 to 1/12800 in serum buffer) against three concentrations of purified LLO (6.25 ng per well to 25 ng per well) in indirect ELISA.

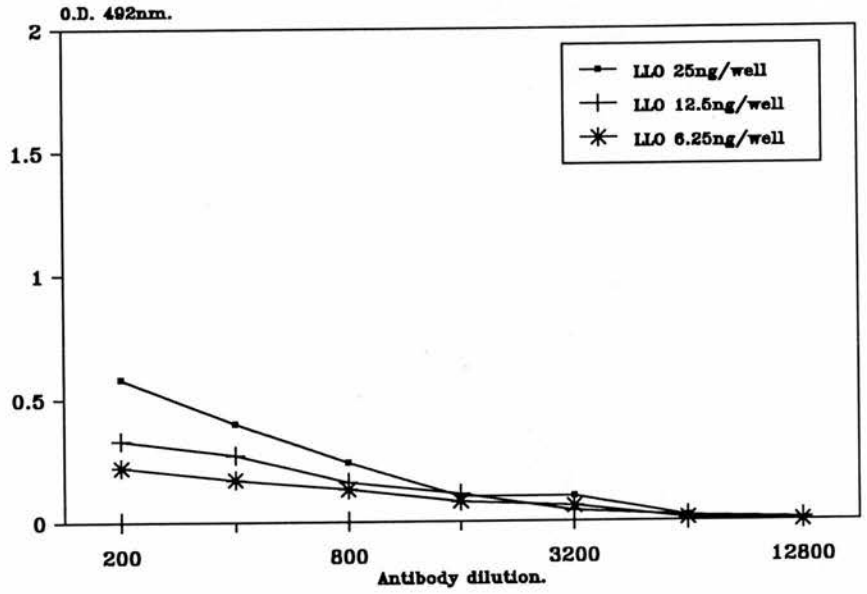


Figure 4.6.

Titration of experimental serum 3 (diluted from 1/200 to 1/12800 in serum buffer) against three concentrations of purified LLO (6.25 ng per well to 25 ng per well) in indirect ELISA.

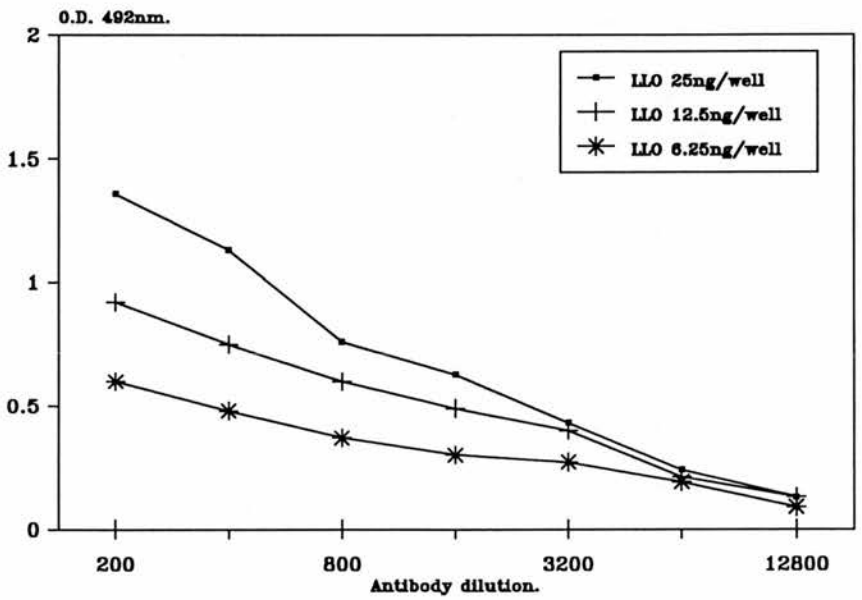
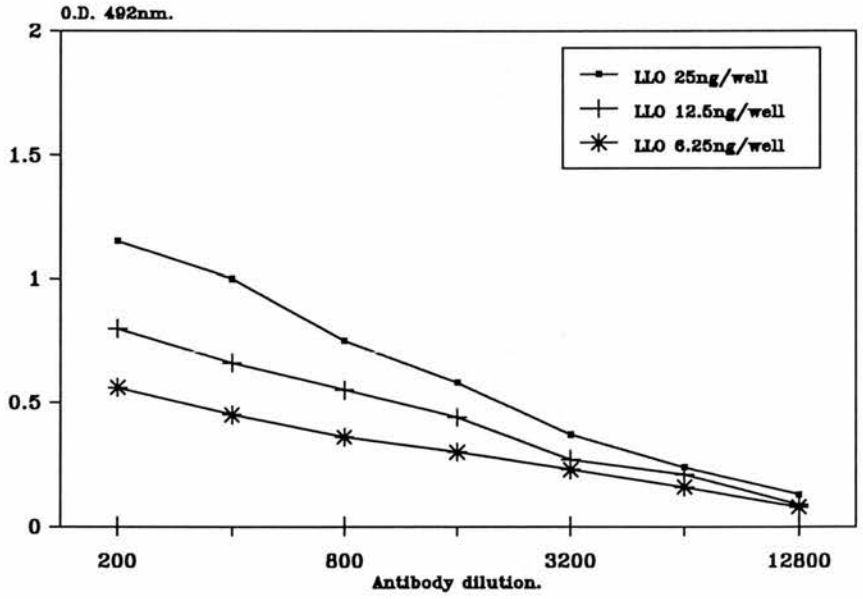


Figure 4.7.

Titration of experimental serum 4 (diluted from 1/200 to 1/12800 in serum buffer) against three concentrations of purified LLO (6.25 ng per well to 25 ng per well) in indirect ELISA.



4.4 Production of a hyperimmune rabbit anti-LLO serum

4.4.1 LLO vaccine preparation

The vaccine was prepared by adding 0.1 ml of sterile 2% (w/v) alhydrogel to 50 μg LLO in 50mM MES buffer, pH 6.5 (chromatography fraction 13), and storing the suspension overnight at 4^{o}C. The buffer containing alhydrogel precipitated LLO was emulsified with an equal volume of bayol/arlacel and the final vaccine was stored at 4^{o}C. The vaccine contained 23 μg protein ml^{-1}.

4.4.2 Hyperimmunization of a rabbit with the LLO vaccine

A pre-immunization bleed was taken from an adult female New Zealand White rabbit. The animal was immunized by the intramuscular injection of 11.5 μg LLO in incomplete adjuvant (section 4.4.1) and a second identical immunization was performed four weeks later. Two months later the animal was injected intravenously with 30 μg LLO in 50mM MES, pH 6.5, buffer. One week later the rabbit was killed and exsanguinated. The blood was allowed to clot overnight and the serum was collected and stored at -20^{o}C.

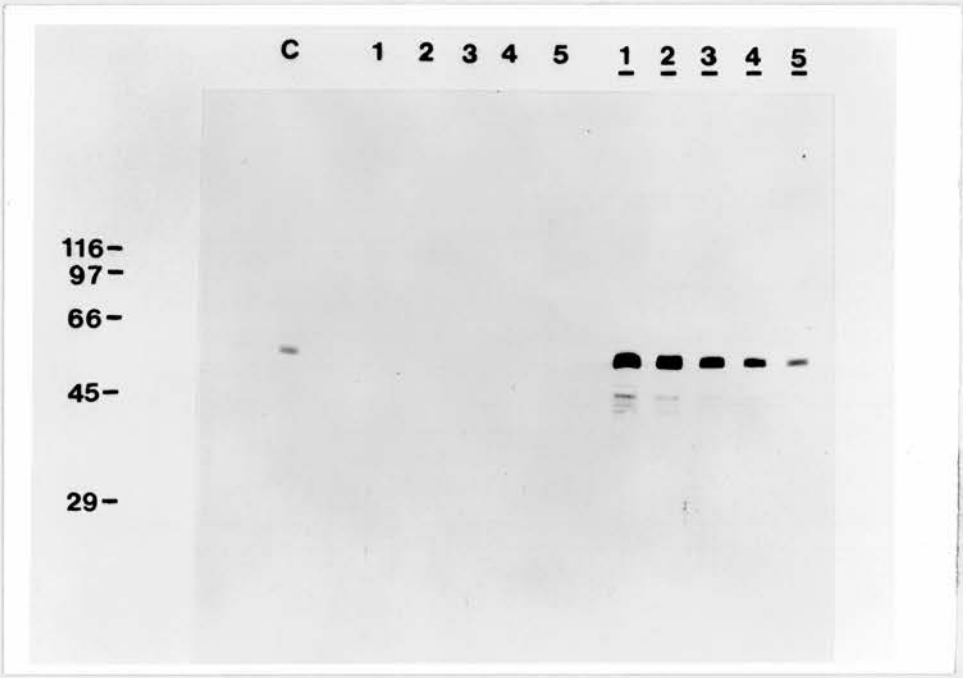
4.4.3 Titration of the hyperimmune anti-LLO serum against LLO in immunoblot

Purified LLO (chromatography fraction 13) was prepared for electrophoresis in SDS-PAGE and loaded as 5 μg protein in a single block well. Following electrophoresis through a 10% resolving gel the protein was transferred to an Immobilon-P membrane (Millipore) by overnight electrophoresis in a wet blot tank (section 2.8.10). Examination for anti-LLO antibodies in the pre and post-immunization rabbit sera (section 4.4.2), titrated from 1/100 to 1/1600 in serum diluent together with a positive control anti-LLO rabbit serum (section 2.9.4), was achieved by immunoblotting (section 2.8.10). The positive control serum was used at a dilution of 1/500 in serum diluent.

The purified LLO was recognized as a 58 kDa protein by the positive control serum but there was no evidence of anti-LLO reactivity in the pre-immunization rabbit serum. Following immunization anti-LLO antibodies were clearly present and the serum was reactive at a 1/1600 dilution (Figure 4.8). Evidence for recognition of a 45 kDa molecular mass contaminant protein was observed with serum dilutions of 1/100 and 1/200.

Figure 4.8.

Titration of the hyperimmune rabbit anti-LLO serum against LLO in immunoblot



Lane C, positive control anti-LLO serum (1/500 dilution). Pre-immunization serum diluted: 1/100 (lane 1); 1/200 (lane 2); 1/400 (lane 3); 1/800 (lane 4); 1/1600 (lane 5). Post-immunization serum diluted 1/100 (lane 1); 1/200 (lane 2); 1/400 (lane 3); 1/800 (lane 4); 1/1600 (lane 5).

Position of molecular mass markers (kDa) shown.

4.5 Examination of the immunological reactivity of the hyperimmune anti-LLO serum with sulphydryl-activated toxins

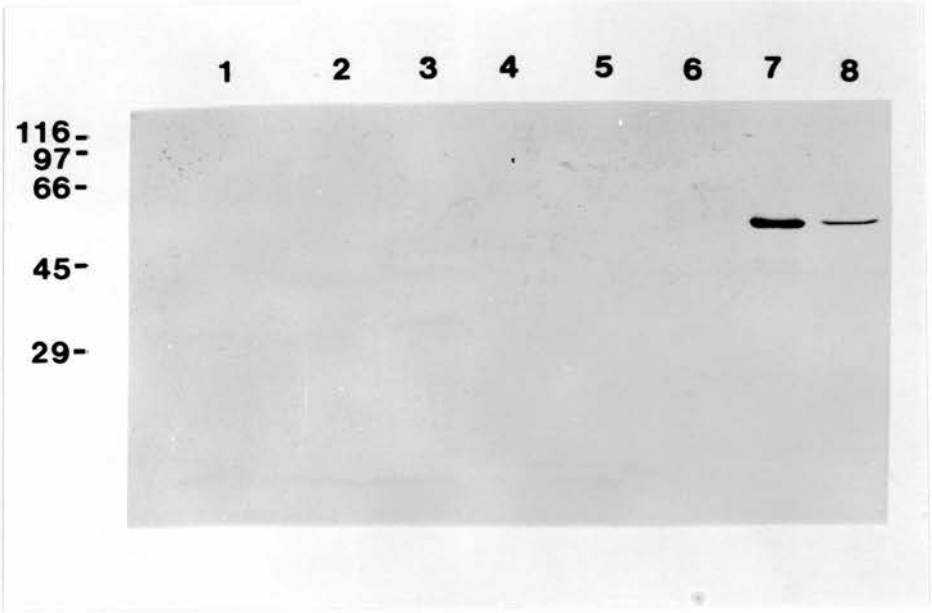
4.5.1 Reactivity of the hyperimmune rabbit anti-LLO serum with sulphydryl-activated toxins in immunoblot

Crude toxin preparations from *Clostridium perfringens*, *Cl. septicum*, *Cl. tetani*, *Cl. novyi* were kindly provided by Hoechst UK Ltd and purified streptolysin O (SLO) was purchased from Sigma. Samples were prepared for SDS-PAGE (section 2.8.7.4) and loaded individually in lanes as: 250 μg of protein for the clostridial toxin preparations, 250 ng purified SLO and 250 ng of purified LLO (chromatography fraction 13). Cholesterol precipitated LLO (section 2.7.3) was loaded as a 10 μl volume. Following electrophoresis through a 10% resolving gel proteins were electrophoretically transferred to an Immobilon-P membrane. The hyperimmune rabbit anti-LLO antiserum, produced in section 4.4 and diluted 1/500 in serum diluent, was blotted against transferred proteins using the method described in section 2.8.10.

The hyperimmune serum recognized a 58 kDa antigen in the purified LLO and cholesterol precipitated LLO preparations (Figure 4.9) but failed to detect an antigen in the clostridial preparations or the purified SLO.

Figure 4.9.

Reactivity of the hyperimmune rabbit anti-LLO serum with sulphydryl-activated toxins in immunoblot



Lane 1, *Cl. perfringens* beta toxin (250 μg); lane 2, *Cl. perfringens* epsilon toxin (250 μg); lane 3, *Cl. septicum* toxin (250 μg); lane 4, *Cl. tetani* toxin (250 μg); lane 5, *Cl. novyi* beta toxin (250 μg); lane 6, SLO (250 ng); lane 7, LLO (250 ng); lane 8, cholesterol precipitated LLO.

Position of molecular mass markers (kDa) shown.

Discussion

In broth cultures haemolysin production peaked after the logarithmic period of growth, which is in agreement with the report by Mengaud *et al.* (1991a). In dialysis sac cultures haemolysin production was found to follow a similar pattern. Though the haemolytic activity was lower in dialysis sac cultures the ease of removal of growth media constituents and the small volume of the concentrated supernatant fluid permitted the purification of a haemolysin in a single cation exchange chromatography step. The haemolysin, collected in fraction 13, possessed the characteristics of LLO (Kreft *et al.*, 1989), was shown in SDS-PAGE to be a single homogeneous protein with a molecular mass of approximately 58 kDa and was recognized in immunoblots by an anti-LLO serum.

An examination of Figures 4.1 and 4.2 revealed a relatively high absorbance value for the chromatogram fraction 12 but no protein content visible upon silver staining. This discrepancy was attributed to the elution of broth pigments which had not been completely removed by dialysis. Though the dialysis sac supernatant fluid contained few proteins visible by silver staining the concentrated supernatant fluid contained many protein

bands which were presumably initially present at undetectable concentrations.

The purification method for LLO was more efficient than any previously published (Geoffroy *et al.*, 1987; Kreft *et al.*, 1989). Considerable amounts of LLO were obtained which remained antigenic and bound efficiently to polystyrene microtitre plates. The optimum concentration of LLO for use in ELISA was determined by titration of antigen against the convalescent serum and was shown to be between 6.25 and 25 ng per well. The optical density produced with an antigen concentration of 25 ng per well and a serum dilution of 1/400 was towards the upper limit of the linear portion of the standard curve. Using the same antigen concentration and antibody dilution each of the experimental sera from infected animals produced optical density values in the range 0.4 to 1.1 and these could be clearly discriminated from the value produced with the serum taken prior to challenge. The standard ELISA conditions for testing of further sera were therefore determined as a 1/400 serum dilution and an antigen concentration for coating of 25 ng LLO per well.

Purified LLO was succesfully used to immunize a rabbit. The resulting hyperimmune serum reacted in immunoblots with LLO and failed to detect SLO or any antigens in the clostridial toxin preparations. Purified SLO was shown

to electrophoretically transfer under the same conditions and was recognized in immunoblots by a hyperimmune anti-SLO antiserum (section 7.2). Though there is no evidence that the crude clostridial preparations contained sulphydryl-activated toxins or that these proteins transferred to the blotting membrane, the results imply that the antigenic cross reactivity shown by the sulphydryl-activated toxins depends upon conformational structure and that specific linear antigenic epitopes exist in LLO. The development of a specific serodiagnostic assay based upon the recognition of LLO is therefore practical and the possibilities of using the LLO ELISA for serodiagnosis and epidemiological investigations were studied in Chapters 6 and 7. With regard to the potential specificity of the ELISA it should be recognized that with the exception of the clostridial species and *L. monocytogenes* the bacteria producing sulphydryl-activated toxins (Alouf and Geoffroy, 1984) are of limited importance in sheep (Buxton and Fraser, 1977; Hardie, 1986).

Experimental Studies

Chapter 5.

Experimental challenge of lambs with *Listeria monocytogenes* and *Listeria innocua*.

Introduction

Listeria species are widespread in the environment having been isolated from soil, fresh water, sewage sludge, decaying vegetation, animal feeds (Gray, 1960; Welshimer, 1968; Welshimer and Donker-Voet, 1971; Weis, 1975a; Watkins and Sleath, 1981; Fenlon, 1985), and the faeces of both apparently healthy and diseased animals (Gray and Killinger, 1966; Rocourt and Seeliger, 1985). Despite the widespread nature of the organism and the presumably frequent occurrence of oral exposure there are few descriptions of clinical responses to oral challenge. The challenge dose at which clinical disease is likely to occur remains unknown and it is unclear whether enteric carriage leads to the development of antibodies to listeric antigens. A series of experiments were therefore conducted to study the clinical responses of sheep to primary oral challenge with live *Listeria monocytogenes*, *L. innocua* and killed *L. monocytogenes*. The animals' serum antibody responses were studied using the immunoassays developed in Chapters 2, 3 and 4.

5.1 Oral and subcutaneous challenge of lambs with viable *Listeria monocytogenes* serovar 4b

5.1.1 Bacteria

L. monocytogenes serovar 4b (NCTC 10527) was used throughout this section. Challenge inocula were prepared as described (section 2.6.2).

5.1.2 Animals

Twelve six month old Suffolk cross Scottish Blackface lambs (section 2.5.3) were randomly allocated to and housed in two separate groups of six.

5.1.3 Experimental protocol

Lambs were dosed orally through tubing passed to the pharynx (section 2.8.15). Group 1 lambs received 5 ml PBS per os on three consecutive days, at the same time Group 2 lambs were given $1x10^{10}$ c.f.u. viable *L. monocytogenes* per day suspended in 5 ml PBS. Over the challenge period and for the next four days clinical examinations were made daily and rectal temperatures recorded.

The level of protective immunity conferred by oral dosing with viable *L. monocytogenes* was assessed by comparing the clinical responses of both groups to a subcutaneous injection, given anterior to the shoulder, of $2x10^{10}$ c.f.u. *L. monocytogenes*. All lambs were challenged 24 days after the first oral dose. Clinical examinations were made and rectal temperatures recorded for seven days after the subcutaneous injections.

Blood samples were cultured, according to the method in section 2.6.3, on days 0 to 6 after the start of oral dosing and after the subcutaneous injections on days 24 to 28. Full postmortem examinations were carried out on two animals after the subcutaneous injections. Samples of brain at the level of the medulla, plus liver, spleen and kidney were taken aseptically and using standard bacteriological techniques inoculated onto 5 per cent sheep blood agar plates. Plates were incubated at 37°C and examined for bacterial growth over 72 h. The samples of medulla were also macerated in 10 ml volumes of TPB (Oxoid), stored at 4°C and subcultured weekly to 5 per cent blood agar plates which were incubated and examined as described above.

Brain, spinal cord, spleen, kidney and liver were collected into phosphate buffered neutral 10 per cent formalin and processed as described in section 2.8.16.

5.1.4 Results of clinical, bacteriological, and pathological examinations

No clinical signs were observed in any lamb during or after the oral dosing, though the mean rectal temperatures of lambs in both groups were raised at 24 h (Table 5.1). At 48 h the mean rectal temperature of Group 2 lambs was significantly higher than that of Group 1 (Student's *t* test, $P<0.05$). At no other time in the period before the subcutaneous injections were significant differences noted.

Lambs in both groups were pyrexic 24 h after the subcutaneous injections (Table 5.2). The rectal temperatures subsided to normal at 48 h in the Group 2 lambs but were elevated for 72 h in the Group 1 lambs. At 48 h after challenge there was a highly significant difference between the two groups (Student's *t* test, $P<0.005$), though the only discernible clinical signs of infection were increased respiratory rates for the duration of pyrexia. The lambs did not become inappetant or develop diarrhoea.

Table 5.1.

Mean rectal temperatures (°C) of lambs after oral dosing with PBS or *L. monocytogenes* on days 0, 1 and 2

	(Group 1, n=6) (PBS) mean rectal temperature±SD.	(Group 2, n=6) (*L. monocytogenes*) mean rectal temperature±SD.
Day 0	39.6±0.6	40.1±0.6
Day 1	40.2±0.6	40.3±0.3
Day 2	39.8±0.5	40.7±0.3*
Day 3	39.6±0.5	39.6±0.7
Day 4	39.1±0.6	39.5±0.6
Day 5	39.5±0.6	39.9±0.4
Day 6	39.7±0.6	39.8±0.3

* Significance in Student's *t* test $P<0.05$.

Forty eight hours after the subcutaneous injections one lamb in Group 2 (case A) was unsteady and showed hindlimb incoordination when moved. The animal's head was not held to either side but was carried unusually low. The rectal temperature was 40.3°C. The clinical condition slowly improved over the following days and when killed 25 days later the lamb was clinically normal. A full postmortem examination was carried out. On day 34, ten days after the subcutaneous injections, another lamb in Group 2 (case B) developed neurological symptoms. This animal when moved showed incoordination of the hindlimbs and a high-stepping hindleg gait. The rectal temperature was 40.9°C. Eight hours later the animal had opisthotonus, was recumbent and making paddling movements with its legs. It was killed and a postmortem examination performed.

L. monocytogenes was not cultured from blood samples taken in the six days from the start of oral dosing. After the subcutaneous challenge *L. monocytogenes* was not isolated from the blood of Group 2 lambs, whereas blood cultures from five of the six lambs in Group 1 were positive on 10 occasions over days 25 to 28. A significant difference in the results of blood culture for the two groups was demonstrated on day 26 (Fisher's exact two tailed test, $P<0.05$) (Table 5.3).

Table 5.2.

Mean rectal temperatures (°C) of lambs after subcutaneous injection with *L. monocytogenes* on day 24

	(Group 1, n=6)	(Group 2, n=6)
	mean rectal temperature±SD.	mean rectal temperature±SD.
Day 23	39.7±0.5	40.0±0.4
Day 24	39.8±0.7	40.1±0.3
Day 25	40.8±0.6	41.0±0.5
Day 26	41.2±0.5**	40.1±0.3
Day 27	40.3±0.8	39.5±0.5
Day 28	39.7±0.4	39.7±0.4
Day 29	39.8±0.5	39.8±0.4
Day 30	39.6±0.3	39.8±0.4
Day 31	39.5±0.3	39.6±0.3

** Significance in Student's *t* test $P<0.005$.

Table 5.3.

Isolation of *L. monocytogenes* from blood samples after subcutaneous injection with *L. monocytogenes* on day 24

	(Group 1, n=6)	(Group 2, n=6)
	positive culture/ number of cultures made	positive culture/ number of cultures made
Day 24	0/6	0/6
Day 25	1/6	0/6
Day 26	5/6*	0/6
Day 27	3/6	0/6
Day 28	1/6	0/6

* Significance in Fisher's exact two tailed test $P<0.05$.

Following one weeks cold enrichment (Gray *et al.*, 1948) *L. monocytogenes* was isolated from the brain of case B, which 10 days after the subcutaneous challenge developed neurological symptoms. *L. monocytogenes* was not isolated from any other tissue and from none of the tissues or brain of case A which was killed 25 days after the subcutaneous challenge.

Postmortem examination revealed no gross lesions in either case A or B but histological lesions were considered to be characteristic for listeric encephalomyelitis (Cordy and Osebold, 1959; Ladds *et al.*, 1974; Charlton and Garcia, 1977). In case A there were microabscesses and lymphocytic perivascular cuffs in the pons, medulla and cervical spinal cord (Plates 5.1 and 5.2). A mild lymphocytic meningitis was also present. The microabscesses featured predominantly macrophage accumulation with local malacia, neuropil vacuolation and axonal degeneration. In the red nucleus of the midbrain chromatolysis of cell bodies was found and in the cervical spinal cord areas of axonal swelling and Wallerian degeneration were seen. No lesions were present in the other tissues examined. In case B the spinal cord was the principal site of the lesions. A single microabscess composed of macrophages and neutrophils was present in the cervical spinal cord (Plate 5.3) and a mild radiculitis affected the ventral

nerve root in the same section. A lymphocytic meningitis extended over the brain, and thick perivascular cuffs composed of lymphocytes were present in the spinal cord and medulla, markedly unilateral in the latter. Extensive hepatocyte vacuolation consistent with fat accumulation was present in the liver (Plate 5.4) and in the kidney proteinaceous deposits were present in Bowman's capsules. A summary of the clinical findings and subsequent bacteriological and pathological results for encephalomyelitis cases A and B is presented in Table 5.4.

Plate 5.1.

Transverse section of the medulla of case A showing microabscessation (small arrow) and axonal degeneration (arrow head). Haematoxylin and eosin X 28.

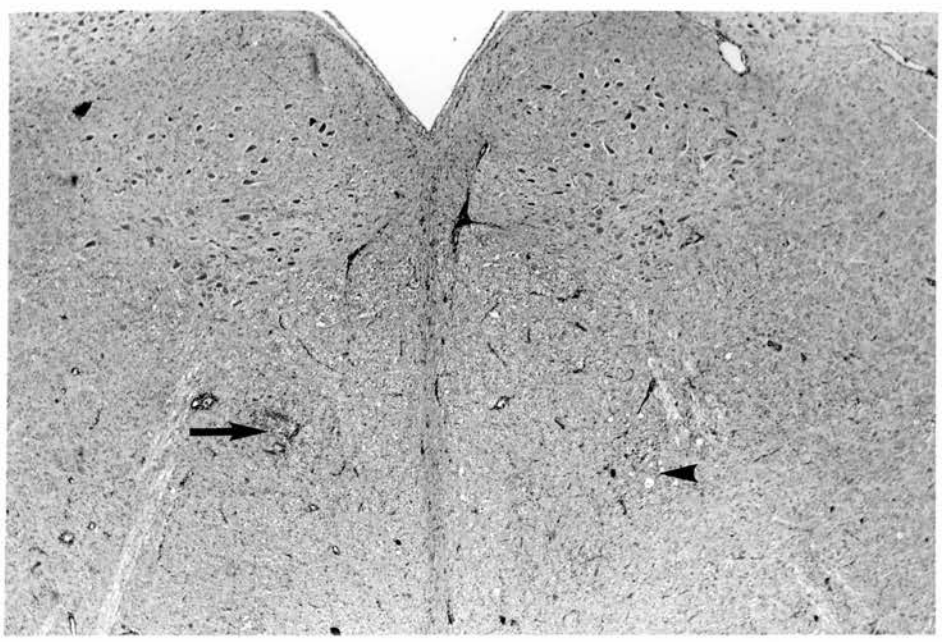


Plate 5.2.

Cervical spinal cord of case A showing axonal degeneration. Haematoxylin and eosin X 280.

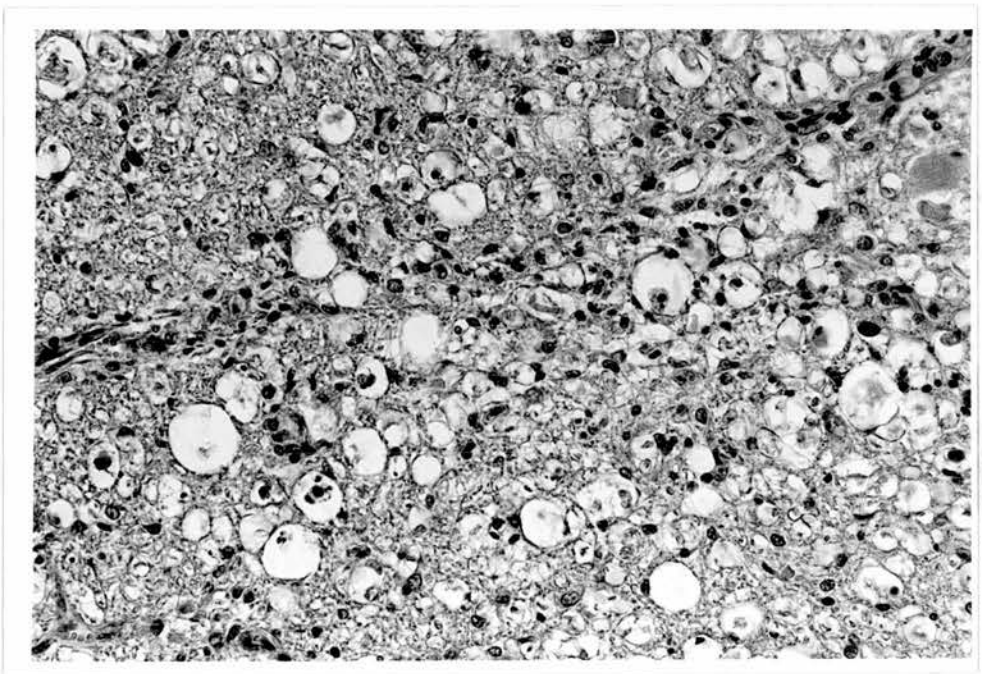


Plate 5.3.

Longtitudinal section of the cervical spinal cord of case B showing microabscessation, perivascular cuffing and meningitis. Haematoxylin and eosin X 28.

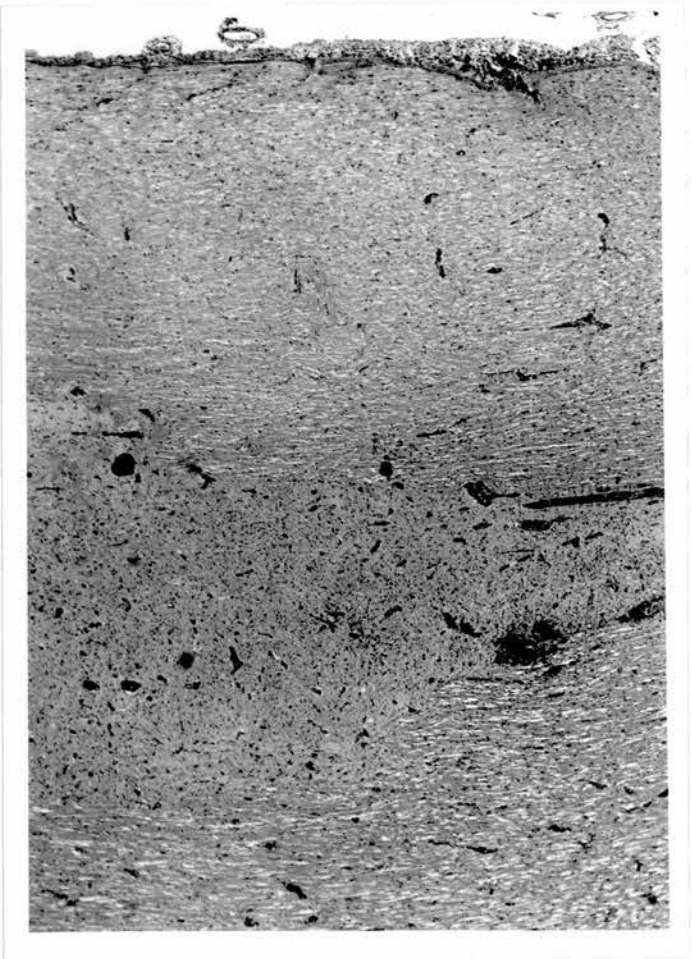


Plate 5.4.

Hepatocyte vacuolation in the liver of case B. Haematoxylin and eosin X 113.

Table 5.4.

Summary of the clinical, bacteriological and pathological findings in encephalomyelitis cases A and B

Case	Group	Rectal temperature	Symptoms	Bacteriology		Pathology	
				Brain	Spinal cord	Medulla	Spinal cord
A (day 26)	2	40.3°C	hindlimb incoordination	-	NT	+	+
B (day 34)	2	40.9°C	hindlimb incoordination	(+)	NT	+	+

\+ positive bacterial culture or presence of pathological lesions
(+) positive bacterial culture after cold enrichment
\- negative bacterial culture or absence of pathological lesions
NT not taken

5.2 Oral dosing of lambs with heat-killed *Listeria monocytogenes* serovar 4b

To determine if passive enteric carriage of *L. monocytogenes* without invasion of the host led to the development of antibodies to listeric antigens it was decided to examine the clinical and serological responses of animals orally dosed with heat-killed *L. monocytogenes*.

5.2.1 Experimental protocol

An aliquot of *L. monocytogenes* serovar 4b (NCTC 10527), prepared as in section 2.6.2, was resuspended in PBS at a concentration of $1x10^{10}$ c.f.u. ml^{-1} and the suspension exposed to moist heat at 100°C for 1 h. Six ten month old Scottish Blackface lambs (Group 3) were fed hay and concentrate. On each day for 3 days these lambs were dosed orally, through tubing passed to the pharynx, with 2 ml of the suspension comprising approximately $2x10^{10}$ c.f.u. killed *L. monocytogenes* serovar 4b. Clinical examinations were made daily and rectal temperatures recorded for seven days.

5.2.2 Clinical responses

Throughout the period the lambs appeared unaffected by the challenge and no increases in rectal temperatures were recorded (Table 5.5).

Table 5.5.

Mean rectal temperatures (°C) of lambs after oral dosing with heat-killed *L. monocytogenes* on days 0, 1 and 2

	(Group 3, n=6) (heat-killed *L. monocytogenes*) mean rectal temperature±SD.
Day 0	39.9±0.5
Day 1	39.6±0.3
Day 2	39.4±0.4
Day 3	39.3±0.3
Day 4	39.3±0.4
Day 5	39.5±0.6
Day 6	39.4±0.3

5.3 Oral dosing of lambs with viable *Listeria monocytogenes* serovar 4b or *Listeria innocua* serovar 6a and subsequent subcutaneous challenge with viable *Listeria monocytogenes* serovar 4b

5.3.1 Bacteria

L. monocytogenes serovar 4b (NCTC 10527) and *L. innocua* serovar 6a (NCTC 11288) were grown and challenge inocula prepared as described in section 2.6.2.

5.3.2 Animals

Eighteen six month old Suffolk cross Scottish Blackface lambs (section 2.5.3) were randomly allocated to three groups of six which were housed separately.

5.3.3 Experimental protocol

Oral doses were given on days 0, 1 and 2 as described in section 5.1.3. Group 4 lambs received 5 ml PBS per os, and Group 5 lambs received in total 3.1×10^9 c.f.u. viable *L. monocytogenes* suspended in PBS. Group 6 lambs were given a total of 4×10^9 c.f.u. viable *L. innocua* suspended in PBS over the same period.

Clinical examinations were made daily and rectal temperatures recorded over the challenge period and for four days afterwards. Blood samples were collected for culture, as described in section 5.1.3, on days 0 to 5. On days 0 to 5 faeces were cultured as described in section 2.6.4.

At 21 days after the oral dosing all lambs were injected on the side of the neck with approximately $8x10^8$ c.f.u. viable *L. monocytogenes* serovar 4b (NCTC 10527) suspended in PBS. Lambs were examined clinically for six days, rectal temperatures were recorded and blood samples cultured on experimental days 21 to 25.

5.3.4 Results of clinical, bacteriological and pathological examinations

As in section 5.1.4 no clinical signs were observed in any of the groups after the oral dosing. However in comparison with the results of section 5.1.4 the lower dose of *L. monocytogenes* did not raise the mean rectal temperatures above 40.2°C at any time (Table 5.6). A one way analysis of variance revealed a significantly higher ($P<0.05$) mean rectal temperature at 72 h in Group 4 (PBS challenge) in comparison with Group 6 (*L. innocua* challenge) but there was no biological sense to this result and at no other time before the subcutaneous injections were significant differences noted.

Table 5.6.

Mean rectal temperatures (^{o}C) of lambs after oral dosing with PBS, *L. monocytogenes* or *L. innocua* on days 0, 1 and 2

	(Group 4, n=6) (PBS) mean rectal temperature$\pm$SD.	(Group 5, n=6) (*L. monocytogenes*) mean rectal temperature$\pm$SD.	(Group 6, n=6) (*L. innocua*) mean rectal temperature$\pm$SD.
Day 0	40.0 ± 0.4	40.1 ± 0.4	39.9 ± 0.2
Day 1	40.1 ± 0.4	39.9 ± 0.2	40.2 ± 0.2
Day 2	39.9 ± 0.6	40.2 ± 0.3	39.8 ± 0.2
Day 3	40.2 ± 0.2*	39.9 ± 0.1	39.8 ± 0.4
Day 4	40.0 ± 0.2	39.9 ± 0.3	40.0 ± 0.2
Day 5	40.0 ± 0.2	40.1 ± 0.2	40.0 ± 0.4
Day 6	40.0 ± 0.3	40.2 ± 0.3	39.9 ± 0.3

* See text for statistical analysis of results.

The subcutaneous injections of $8x10^8$ c.f.u. of *L. monocytogenes* resulted in no marked pyrexia at 24 h (Table 5.7). However, one way analysis of variance revealed the mean rectal temperature of the control Group 4 to be significantly higher ($P<0.05$) than that of Group 5 previously challenged with *L. monocytogenes*. At 48 h the mean rectal temperature in Group 5 was significantly lower in comparison to both Groups 4 ($P<0.001$) and 6 ($P<0.05$), and at 72 h significantly lower than the mean rectal temperature for Group 4 ($P<0.05$). The only discernible clinical signs were increased respiratory rates in Groups 4 and 6 for 72 h after the subcutaneous injections. As in section 5.1.4 the lambs were not inappetant and did not develop diarrhoea.

Table 5.7.

Mean rectal temperatures (^{o}C) of lambs after subcutaneous injection with *L. monocytogenes* on day 21

	(Group 4, n=6) mean rectal temperature$\pm$SD.	(Group 5, n=6) mean rectal temperature$\pm$SD.	(Group 6, n=6) mean rectal temperature$\pm$SD.
Day 21	40.2 ± 0.3	40.0 ± 0.2	40.1 ± 0.2
Day 22	40.6 ± 0.4*	40.0 ± 0.3	40.4 ± 0.5
Day 23	40.7 ± 0.6***	39.4 ± 0.2	40.1 ± 0.5*
Day 24	40.0 ± 0.7*	39.3 ± 0.3	39.9 ± 0.4
Day 25	39.8 ± 0.5	39.6 ± 0.3	39.9 ± 0.5
Day 26	39.8 ± 0.3	39.6 ± 0.4	40.0 ± 0.3

* See text for statistical analysis of results.

On experimental day 47 a lamb in Group 6 was found dead (case C). Two days later a lamb in Group 4 (case D) was unsteady, the animal's head was held low, and the hind legs were held wide apart. The animal was dull and the rectal temperature was 40.3°C. This animal was killed immediately. On day 55 a lamb in Group 5 (case E) developed a flaccid paralysis of the right foreleg, no pain perception was evident in the limb and there were no other clinical signs. The rectal temperature was 39.6°C and the animal was killed. Sixty one days after the subcutaneous injections a lamb in Group 6 (case F) developed neurological symptoms, no clinical examination was made and the lamb was killed. Postmortem examinations were carried out on all these cases and tissues were taken for histological and bacteriological examinations (section 5.1.3). Additionally, the cervical spinal cords were subjected to bacterial culture and histopathological examinations included sections of the cervical, thoracic and lumbar spinal cords.

No *L. monocytogenes* or *L. innocua* were isolated from blood samples taken in the six days after the oral dosing, and no *Listeria* spp. were detected on culture of faeces on days 0 to 5. Using the method described the minimum numbers of *Listeria* spp. detectable on faecal culture were approximately 5×10^2 c.f.u. g^{-1}.

Table 5.8.

Isolation of *L. monocytogenes* from blood samples after subcutaneous injection with *L. monocytogenes* on day 21

	(Group 4, n=6) positive culture/ number of cultures made	(Group 5, n=6) positive culture/ number of cultures made	(Group 6, n=6) positive culture/ number of cultures made
Day 21	0/6	0/6	0/6
Day 22	2/6	0/6	2/6
Day 23	1/6	1/6	2/6
Day 24	2/6	0/6	2/6
Day 25	2/6	1/6	0/6

No significant difference in Fisher's exact two tailed test.

No significant difference was found in the results of blood cultures after the subcutaneous injection of *L. monocytogenes*. The organism was isolated from the blood of five lambs in Group 4, and four in Group 6 over days 22 to 25 (Table 5.8). The single animal with a demonstrable bacteraemia in Group 5 had not seroconverted to listeric somatic antigens or to LLO after the oral challenge with *L. monocytogenes* (see Chapter 6 for serology results).

L. monocytogenes was isolated on direct culture of the cervical spinal cord and medulla oblongata of case C but from no other tissues. *L. monocytogenes* was not isolated from the brain or cervical spinal cord of case D on direct culture or after 8 weeks cold enrichment, and *L. monocytogenes* was not isolated from the other tissues upon direct culture. The cervical spinal cord of case E yielded *L. monocytogenes* after one weeks cold enrichment, but the organism was not isolated from the brain after six weeks cold enrichment or on direct culture of other tissues. Only the brain was cultured from case F and *L. monocytogenes* was isolated directly.

No gross lesions were apparent in cases C, D, E or F, though histological lesions were characteristic of listeric encephalomyelitis. In case C a marked meningitis extended over the spinal cord and brain stem. Severe inflammatory changes were present in the cervical spinal cord with thick lymphocytic perivascular cuffs, extensive malacia, neuropil vacuolation, and microabscesses featuring both neutrophils and mononuclear cells (Plates 5.5 and 5.6). A marked lymphocytic neuritis affected spinal nerves in the same section (Plate 5.7). Similar lesions were present to a lesser degree in the thoracic and lumbar spinal cord, were bilateral in the medulla, but were not apparent in the mid brain. No lesions were found in the other tissues.

Plate 5.5.

Medulla of case C showing degeneration of axons and a microabscess with neutrophil and mononuclear cell involvement. Haematoxylin and eosin X 280.

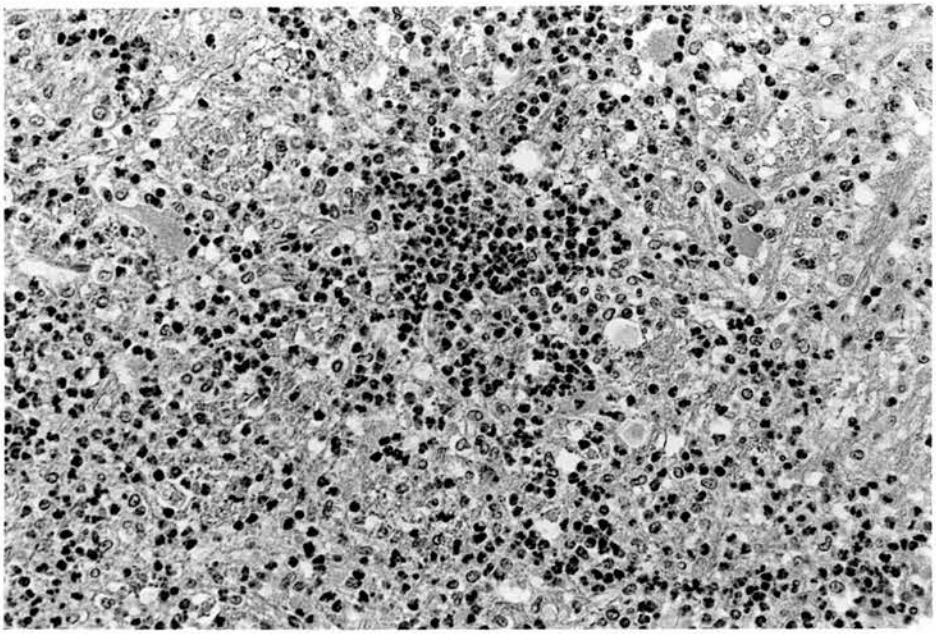


Plate 5.6.

Transverse section of the cervical spinal cord of case C showing severe myelitis with microabscesses and prominent perivascular cuffs. Haematoxylin and eosin X 28.

Plate 5.7.

Spinal nerve of case C affected by severe lymphocytic neuritis. Haematoxylin and eosin X 113.

In case D microabscesses, with both neutrophil and macrophage involvement, were present in the cervical spinal cord and bilaterally in the medulla. A mild neuritis affected spinal nerves in the cervical cord and mild lymphocytic perivascular cuffing and meningitis were present rostrally to the midbrain (Plates 5.8 and 5.9). There was neuropil degeneration, vacuolation and neuronophagia of neurones together with chromatolysis of cell bodies in the red nucleus. In the kidney microabscesses were present in the cortex (Plate 5.10) and in the liver the numbers of lymphocytes were increased in periportal areas. A severe lymphocytic meningitis extended from the cervical spinal cord to the midbrain in case E. Severe inflammatory changes were also present in the cervical cord with marked perivascular cuffing and neuropil degeneration and vacuolation (Plate 5.11). Microabscesses were becoming confluent and consisted predominantly of mononuclear cells. There was axonal degeneration and a heavy infiltration by inflammatory cells in the spinal nerves of the same section. In caudal sections of the spinal cord lesions consisted of perivascular cuffing and axonal degeneration. No inflammatory lesions were present within the medulla or the midbrain though occasional chromatolysis of cell bodies was apparent. No lesions were found in the liver, spleen or kidney.

Plate 5.8.

Transverse section of the medulla of case D showing a microabscess with both neutrophil and mononuclear cell involvement and the edge of a thick perivascular cuff. Haematoxylin and eosin X 280.

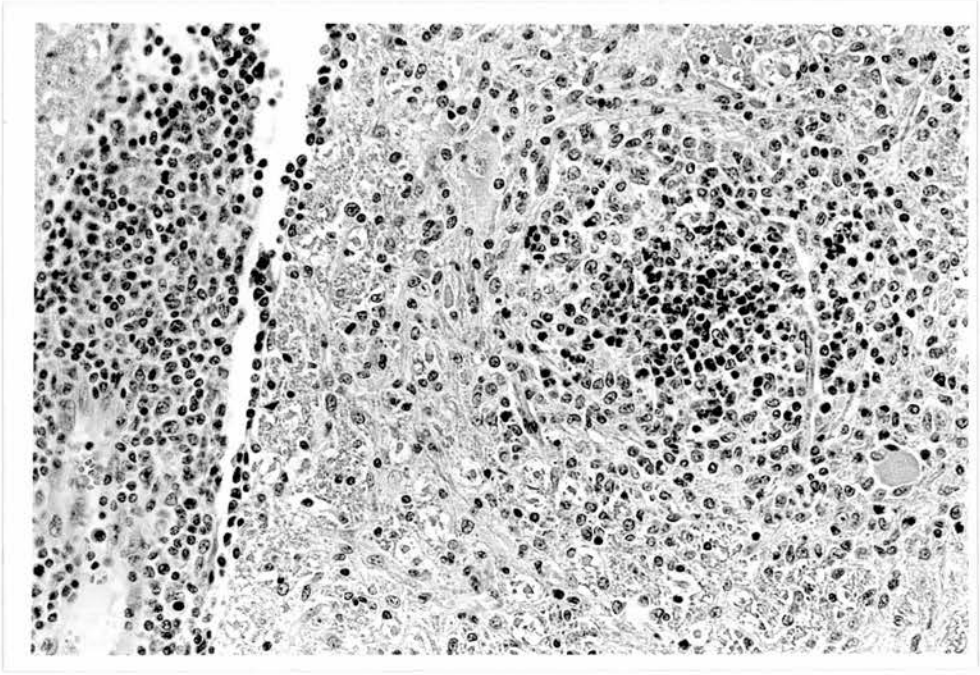


Plate 5.9.

Transverse section of the cervical spinal cord of case D showing mild myelitis. Haematoxylin and eosin X 28.

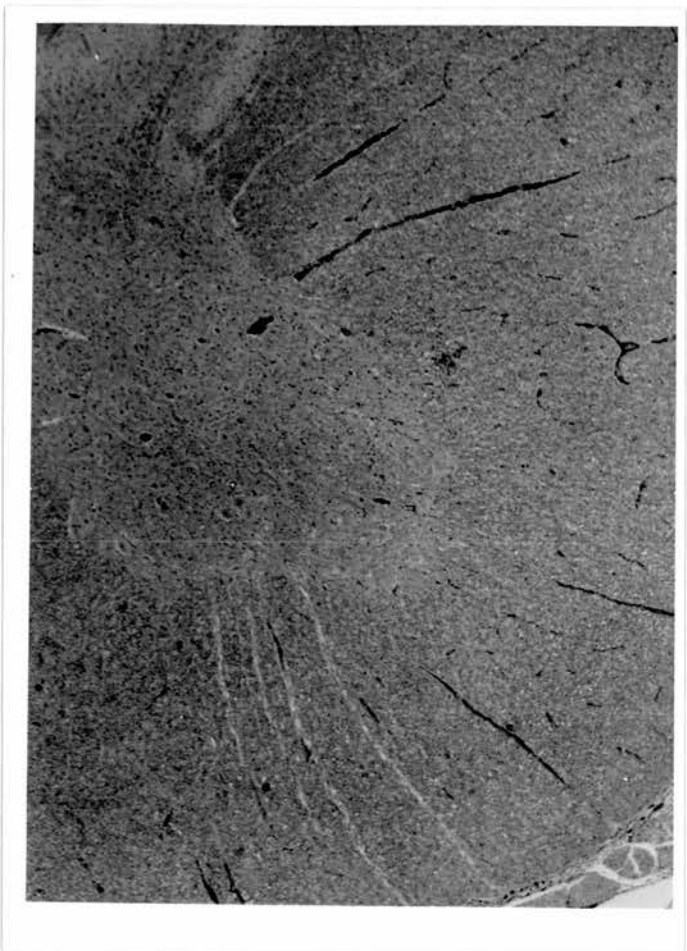


Plate 5.10.

Focal microabscess in the kidney cortex of case D. Haematoxylin and eosin X 113.

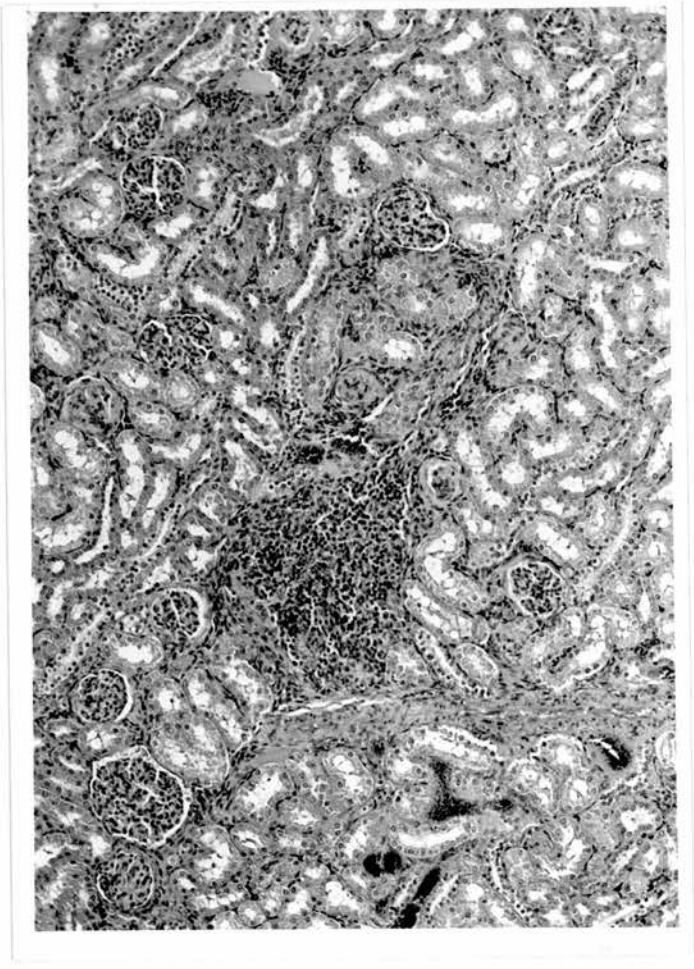


Plate 5.11.

Transverse section of the cervical spinal cord of case E showing a severe myelitis with a marked radiculitis. Haematoxylin and eosin X 28.

In case F only the brain was examined histologically. Mild lymphocytic perivascular cuffing and microabscesses featuring predominantly polymorph leucocytes were present in the medulla and a lymphocytic meningitis extended over the medulla and midbrain (Plate 5.12). A summary of the clinical findings and subsequent bacteriological and pathological results for encephalomyelitis cases C, D, E and F is presented in Table 5.9.

Plate 5.12.

Transverse section of the medulla of case F showing lymphocytic perivascular cuffing and marked microabscessation. Haematoxylin and eosin X 113.

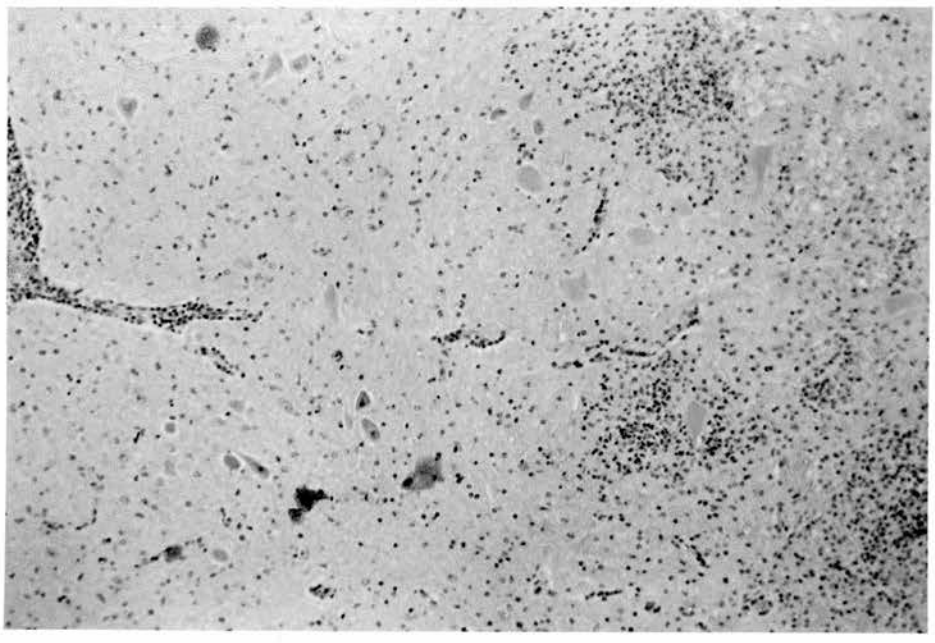


Table 5.9.

Summary of the clinical, bacteriological and pathological findings in encephalomyelitis cases C, D, E and F

Case	Group	Rectal temperature	Symptoms	Bacteriology		Pathology	
				Brain	Spinal cord	Medulla	Spinal cord
C (day 47)	6	NT	dead	+	+	+	+
D (day 49)	4	40.3°C	hindlegs held wide apart	-	-	+	+
E (day 55)	5	39.6°C	paralysis of right foreleg	-	(+)	-	+
F (day 61)	6	NT	unrecorded	+	NT	+	NE

\+ positive bacterial culture or presence of pathological lesions
(+) positive bacterial culture after cold enrichment
\- negative bacterial culture or absence of pathological lesions
NT not taken
NE not examined

Discussion

Oral dosing of lambs with either $3.1x10^9$ or $3x10^{10}$ c.f.u. viable *L. monocytogenes* serovar 4b resulted in no apparent clinical illness. However, in comparison to control animals the higher dosage resulted in pyrexia on Day 2. The lack of a clinical response following the oral dosing is in agreement with Paterson (1940a) who produced no clinical symptoms in ewes given higher doses of the organism and Graham *et al.* (1940a) who recorded pyrexia of short duration following the oral dosing of a single lamb. Osebold and Inouye (1954b) reported depression, inappetance and pyrexia in sheep given higher doses of the organism, though they comment that by cursory examination there was no apparent infection.

Using other species of experimental animals, Farber *et al.* (1991) described symptoms of septicaemia and reported 48 h to be the peak of pyrexia after orally dosing cynomolgus monkeys with 10^9 c.f.u. *L. monocytogenes* serovar 4b. Similarly Miettinen *et al.* (1990) reported febrile illness 1 to 2 days after orally dosing adult goats with $6x10^9$ c.f.u. *L. monocytogenes* serovar 1/2a. The symptoms described included pyrexia, inappetance and reduced milk yields but four of the five goats made complete clinical recoveries without treatment.

A highly significant reduction in the number of positive blood cultures and significantly lower rectal temperatures in lambs previously dosed with $3x10^{10}$ c.f.u. *L. monocytogenes* indicated that oral challenge with viable organisms resulted in the development of immunity to the subsequent subcutaneous challenge. This result is in agreement with a report by MacDonald and Carter (1980) in which a primary oral challenge of mice resulted in protection against secondary oral or intravenous challenge. In mice the development of protective immunity is dependant upon challenge by live *L. monocytogenes* (Graham *et al.*, 1940b; Mackaness, 1962; Coppel and Youmans, 1969; Wirsing von Koenig *et al.*, 1982; 1983) with tissue multiplication in the host (North *et al.*, 1981; Kaufmann, 1984b; Berche *et al.*, 1987a). The results suggest that after the oral dosing of lambs invasion and systemic infection occurred even though no organisms were cultured from the blood. The occurrence of pyrexia at 48 h is within the period of greatest bacterial multiplication in murine tissues after oral dosing (MacDonald and Carter, 1980; Roll and Czuprynski, 1990) and the consistent seroconversion of lambs in Group 2 to listeric antigens (Chapter 6) is further support for the suggestion that systemic infection occurred after oral dosing.

Racz *et al.* (1972) have described the ability of *L. monocytogenes* to enter intestinal epithelial cells after oral dosing of guinea-pigs and *in vitro* the organism is capable of invading and spreading from cell to cell in various cell lines including Caco2 enterocytes (Gaillard *et al.*, 1987; Tilney and Portnoy, 1989; Kuhn and Goebel, 1989; Mounier *et al.*, 1990). It is suspected that the oral dosing of lambs with $3x10^{10}$ c.f.u. *L. monocytogenes* resulted in invasion and multiplication of the organism and possibly dissemination via the mesenteric lymph nodes to the liver as has been described in mice and rats (Audurier *et al.*, 1980; 1981; MacDonald and Carter, 1980; Schlech *et al.*, 1986; Roll and Czuprynski, 1990). The failure to isolate the organism from the blood samples may be explained by its entrapment in cells of the reticuloendothelial system (Mackaness, 1962) or circulating organisms may have been lethally damaged within blood monocytes.

Host invasion following the lower dosage used in section 5.3 was apparently inconsistent. No pyrexia was recorded after oral dosing and following the subcutaneous challenges one animal in Group 5 became bacteraemic. The animals' serological responses were variable and convincing seroconversion to listeric antigens was only demonstrable in four lambs (Chapter 6). The one Group 5 animal in which there was clearly no seroconversion became bacteraemic after the subcutaneous challenge.

After the subcutaneous challenges the rectal temperatures of Group 5 lambs were significantly lower than those of Groups 4 and 6 at 48 hours but the failure to detect a bacteraemia in all the Group 4 lambs and development of a bacteraemia in one Group 5 lamb rendered impracticable a definition of the degree of protection which followed oral dosing with *L. innocua*. However, the absence of a serological response following oral dosing with *L. innocua* (Chapter 6) and a tendency for the blood culture results after the subcutaneous challenges to resemble those of the Group 4 control lambs suggests that the Group 6 lambs developed no significant protection to the subsequent subcutaneous challenge with *L. monocytogenes*.

The findings reproduce results in mice, rats and monkeys where numbers ingested are important in the establishment of infection and a relatively large challenge, in the order of 10^8 to 10^9 c.f.u. is required to consistently initiate systemic infection in these species (Audurier *et al.*, 1980; MacDonald and Carter, 1980; Schlech, 1990; Farber *et al.*, 1991). It may be deduced that for *L. monocytogenes* serovar 4b (NCTC 10527) approximately 10^{10} c.f.u. is the oral dose which results in reliable systemic infection of lambs.

In the five days after oral challenge the bacteriological examinations failed to demonstrate faecal excretion of more than $5x10^2$ c.f.u. *Listeria* spp. in any animal. Similarly, MacDonald and Carter (1980) reported that oral dosing of mice did not produce enteric carriage rates higher than $10^{2.5}$ c.f.u. and Roll and Czuprynski (1990) report that after intragastric inoculation *L. monocytogenes* were eliminated from the gastrointestinal tract of mice within 4 to 6 days. The results do not contradict those of Farber *et al.* (1991) in which monkeys orally dosed with 10^9 c.f.u. *L. monocytogenes* excreted the organism in their faeces for up to 23 days since these authors used a selective, enrichment medium for the isolation procedure and the bacterial numbers were not enumerated. The results of the present study are in contrast to those of Miettinen *et al.* (1990) who found

faecal excretion rates of 10^{10} c.f.u. g^{-1} after oral dosing of goats with $6x10^9$ c.f.u. *L. monocytogenes*.

These authors used an isolation technique similar to the method described in section 5.3.3 but enumerated *Listeria* solely by their appearance upon listeria selective media (Oxford formulation). Their results must be regarded with some caution as *Streptococci*, *Bacillus* spp. or other *Listeria* spp. may grow on this medium and must be distinguished from *L. monocytogenes*. The clinical signs and serological responses described by these authors are similar to those presented in sections 5.1.4, 5.3.4 and Chapter 6. However, the conflicting results regarding faecal excretion may be due to: the described differences in experimental method, the result of challenge with strains of differing virulence or the consequence of differences in the management, age or species of experimental animal.

The primary objective of this work was the examination of clinical responses to experimental challenge and an evaluation of serological assays for the measurement of humoral antibody responses to listeric antigens. Though postmortem examinations of all the animals were not performed examinations were carried out on six lambs which developed neurological symptoms after the subcutaneous injections. Lesions were typical of

listeric encephalomyelitis but the distribution of lesions was unusual with the cervical spinal cords principally affected. These finding were intriguing since in natural cases spinal cord lesions are always of lesser intensity to those in the brain stem (Cordy and Osebold, 1959; Ladds *et al.*, 1974; Charlton and Garcia, 1977). The clinical findings, histological lesions and bacteriological results are consistent with the rare reports of listeric myelitis (Gates *et al.*, 1967; Seaman *et al.*, 1990). The pathogenesis of the condition is unclear but it seems probable that injection on the side of the neck led to the development of lesions in the spinal cord as a result of the organism ascending via the spinal nerves following this unnatural route of infection.

Chapter 6.

The serological responses of experimentally challenged lambs examined in a variety of immunoassays.

Introduction

There are no satisfactory serological tests for the diagnosis of listeric infections and epidemiological studies are hampered by the poor specificity of the currently available assays. In the development of novel diagnostic tests it is important to clarify whether serum antibodies to listeric antigens develop as a result of the passive enteric carriage of *Listeria* spp. or only through infection of the host. Although the definition of infection as the presence of an organism in or on an animal would strictly include the presence of *L. monocytogenes* in the bowel it is considered that this definition is inappropriate for an environmental organism which can be present in foodstuffs and has been isolated from the intestinal contents of apparently healthy animals. It is therefore suggested that after oral dosing with the organism "infection" should refer to invasion of host tissues. Any assay for listeric infections must have a high specificity to avoid a high

proportion of false positive results in the diagnosis of a disease with a low prevalence.

In the previous chapters novel immunoassays were developed and the clinical responses of lambs experimentally challenged with *Listeria* spp. were examined. In this chapter these animals were categorised as "infected" and "uninfected" and sera derived from these experiments were used to determine the sensitivity of the diagnostic assays.

6.1 Serological responses of experimentally challenged lambs to listeric antigens

6.1.1 Experimental protocol

The clinical responses of lambs to oral dosing and subcutaneous challenge with viable *L. monocytogenes* serovar 4b and *L. innocua* serovar 6a were described in sections 5.1 and 5.3. Similarly the clinical responses of lambs following oral dosing with heat-killed *L. monocytogenes* serovar 4b were described in section 5.2. In this chapter the humoral antibody responses of these experimentally challenged animals to crude and defined listeric antigens were examined using novel immunoassays developed in Chapters 2, 3 and 4.

Serum from animals in experimental Groups 1 and 2 were collected from blood samples taken on days 0, 7, 14 and 21 after oral dosing, and days 11, 25, 39 after subcutaneous challenge with *L. monocytogenes* (i.e experimental days 35, 49 and 63). Serum samples were collected from blood samples taken from the Group 3 lambs on experimental days 0, 7, 14, 21, 28 and 42 and from the Group 4, 5 and 6 lambs 21 days after oral dosing and 14, 28 and 42 days after the subcutaneous injection with *L. monocytogenes* (i.e. experimental days 35, 49 and 63).

The experimental sera were tested for antibodies to listeric somatic antigens in serum agglutination tests (section 2.8.5) and to whole heat-killed cells of *L. monocytogenes* serovar 4b (NCTC 10527) by indirect ELISA (section 2.8.3.1). A competitive sandwich ELISA with PE2 as antigen (section 2.8.3.2) was used to measure the serological responses of Groups 1 and 2 to the putative LTA antigen. The lambs serological responses to LLO were examined in immunoblots using cholesterol precipitated LLO (section 2.7.3) as described by Low and Donachie (1991). In these immunoblots the positive control serum was either a hyperimmune anti-LLO rabbit serum (section 2.9.4) used at a dilution of 1/500 in serum diluent or the convalescent goat serum (section 2.9.3) used at a dilution of 1/100. The test sheep sera were diluted 1/100 in serum diluent. The antibody isotypes involved

in the anti-LLO responses of two challenged and two control lambs were determined using mouse monoclonal antibodies to ovine IgM and IgG_1 (section 2.9.5). These murine antibodies were used as intermediate antibodies following incubation of the immunoblots with the test sheep sera and necessitated the use of anti-mouse immunoglobulin HRP conjugates.

6.1.2 Measurement of antibodies to somatic antigens in serum agglutination tests and whole cell indirect ELISA

In Group 2 the antibody responses to somatic antigens were qualitatively similar in both the SAT (Table 6.1) and whole cell ELISA (Table 6.2). In both assays antibodies were detected in the sera of Group 2 animals on days 14 and 21 after oral dosing with *L. monocytogenes*. At this time the Group 1 control lambs had no measurable antibody response to *L. monocytogenes* in either assay. The assays also revealed that antibodies to somatic antigens were detectable in all lambs of Groups 1 and 2 after subcutaneous injection with the organism. The oral dosing of Group 3 lambs with heat-killed *L. monocytogenes* produced no increase in SAT antibody titres (result not shown) and the whole cell ELISA revealed no evidence of seroconversion (Table 6.3).

The serological responses of lambs in Groups 4, 5 and 6 to somatic antigens determined by whole cell ELISA are shown in Table 6.4. At day 21, after oral dosing with $3.1x10^9$ c.f.u. *L. monocytogenes*, marked increases in percentage absorbance values were demonstrated in four lambs in Group 5, with a weak response in one other lamb and no increase in the sixth. At the same time, after oral dosing with PBS or *L. innocua*, lambs in control Groups 4 and 6 showed no evidence of seroconversion to *Listeria* somatic antigens. The subcutaneous injection of *L. monocytogenes* resulted in the seroconversion of control animals and the percentage absorbance values were maintained in all groups to at least day 63.

Table 6.1.

Geometric mean reciprocal titres of Groups 1 and 2 to somatic antigens (Behringwerke) measured in tube agglutination tests

Day	Group 1 (n=6)	Group 2 (n=6)
	(Antibody titre expressed as mean reciprocal titre).	
0	28	25
7	NT	NT
14	32	646
21	32	500
35	1050	646
49	1050	240 (n=5)

Group 1 dosed orally with PBS on days 0, 1 and 2, and injected subcutaneously with $2x10^{10}$ c.f.u. viable *L. monocytogenes* on day 24. Group 2 dosed orally with $3x10^{10}$ c.f.u. viable *L. monocytogenes* over days 0, 1 and 2, and injected subcutaneously with $2x10^{10}$ c.f.u. on day 24.

Table 6.2.

Serological responses of Groups 1 and 2 to whole heat-killed cells of *L. monocytogenes* measured by indirect ELISA

Day	Group 1 (n=6)	Group 2 (n=6)
	(Antibody titre expressed as mean % absorbance ± SE).	
0	2.7±0.9	5.0±1.1
7	3.6±1.3	2.3±1.1
14	4.1±1.3	30.8±12.2
21	7.1±1.3	70.7±9.4
35	86.8±5.1	94.7±1.2
49	83.0±4.6	85.2±6.9 (n=5)

Test serum dilution 1/100. Group 1 dosed orally with PBS on days 0, 1 and 2, and injected subcutaneously with $2x10^{10}$ c.f.u. viable *L. monocytogenes* on day 24. Group 2 dosed orally with $3x10^{10}$ c.f.u. viable *L. monocytogenes* over days 0, 1 and 2, and injected subcutaneously with $2x10^{10}$ c.f.u. on day 24.

Table 6.3.

Serological responses of Group 3 to whole heat-killed cells of *L. monocytogenes* measured by indirect ELISA

Day	Group 3 (n=6)
	(Antibody titre expressed as mean % absorbance ± SE).
0	10.7±2.5
7	8.8±3.3
14	7.0±2.2
21	6.2±1.6
28	6.3±1.8

Test serum dilution 1/100. Group 3 dosed orally with $2x10^{10}$ c.f.u. heat-killed *L. monocytogenes* on days 0, 1 and 2.

Table 6.4.

Serological responses of Groups 4, 5 and 6 to whole heat-killed cells of *L. monocytogenes* measured by indirect ELISA

Day	Group 4 (n=6)	Group 5 (n=6)	Group 6 (n=6)
	(Antibody titre expressed as mean % absorbance ± SE).		
0	15.0±1.2	15.1±2.3	15.1±1.2
7	NT	NT	NT
14	NT	NT	NT
21	15.5±2.2	68.5±16.0	12.7±1.8
35	92.8±0.8	95.1±2.8	93.7±2.7
49	92.2±2.9	89.7±4.3	91.3±2.5 (n=5)
63	90.1±2.0 (n=5)	93.3±4.5 (n=5)	92.1±9.4 (n=4)

Test serum dilution 1/100. Group 4 dosed orally with PBS on days 0, 1 and 2, and injected subcutaneously with $8x10^8$ c.f.u. viable *L. monocytogenes* on day 21. Group 5 dosed orally with $3.1x10^9$ c.f.u. viable *L. monocytogenes* over days 0, 1 and 2, and injected subcutaneously with $8x10^8$ c.f.u. on day 21. Group 6 dosed orally with $4x10^9$ c.f.u. viable *L. innocua* over days 0, 1 and 2, and injected subcutaneously with $8x10^8$ c.f.u. viable *L. monocytogenes* on day 21.

6.1.3 Results of a competitive sandwich ELISA for the detection of anti-LTA antibodies

The serological responses to the putative LTA measured by competitive sandwich ELISA were weak. At day 14 slight inhibition of the standard was seen with Group 2 sera though by day 21 there was no difference between the anti-LTA antibody titres of Group 2 challenge animals and Group 1 control animals (Table 6.5). An increase in % inhibition value was detected in both groups on experimental day 35 but individuals showed considerable variation and on day 49 the antibody responses were waning. The assay was considered unsuitable as a diagnostic test and the serological responses of lambs in experimental groups 3, 4, 5 and 6 were therefore not examined by this technique.

Table 6.5.

Serological responses of Groups 1 and 2 to LTA measured by competitive sandwich ELISA

Day	Group 1 (n=6)	Group 2 (n=6)
	(Antibody titre expressed as mean % inhibition ± SE).	
0	13.6±3.2	13.4±1.6
7	17.8±4.3	20.4±3.0
14	19.6±1.9	26.6±1.3
21	19.4±4.6	16.9±3.7
35	52.6±13.8	51.5±6.3
49	27.3±5.2	32.9±10.4 (n=5)

Test serum dilution 1/2. Group 1 dosed orally with PBS on days 0, 1 and 2, and injected subcutaneously with $2x10^{10}$ c.f.u. viable *L. monocytogenes* on day 24. Group 2 dosed orally with $3x10^{10}$ c.f.u. viable *L. monocytogenes* over days 0, 1 and 2, and injected subcutaneously with $2x10^{10}$ c.f.u. on day 24.

6.1.4 Detection of antibody responses to LLO by immunoblotting

Cholesterol precipitated LLO was used as the antigen in immunoblotting studies. Antibodies to a 58 kDa antigen, which was also recognized by the control anti-LLO serum, were detected in Group 2 lambs on day 21 after oral dosing with $3x10^{10}$ c.f.u. viable *L. monocytogenes*. No anti-LLO antibodies were detected in any prebleed or in the Group 1 control lambs after dosing with PBS (Figure 6.1). Following the subcutaneous injection of *L. monocytogenes* all the lambs had detectable anti-LLO antibodies and these were maintained to at least day 63. After both the oral dosing and subcutaneous challenges the isotype of antibody recognizing LLO was shown to be predominantly IgG_1 (Figure 6.2). The oral dosing of lambs with heat-killed *L. monocytogenes* produced no anti-LLO antibodies in sera taken on days 14 or 21 (Figure 6.3).

At day 21 after oral dosing with PBS or with viable *L. innocua* no recognition of LLO was demonstrable with sera from lambs of Groups 4 or 6. The results for Group 5 lambs, following oral dosing with $3.1x10^9$ c.f.u. *L. monocytogenes*, revealed similar antibody responses to those obtained by whole cell ELISA (section 6.1.2). At day 21 anti-LLO antibodies were detected in four animals,

there was a weak response by one lamb, and anti-LLO antibodies were undetectable in the sixth (Figure 6.4). After the subcutaneous challenge anti-LLO antibodies were detected in all animals on experimental days 35 and 49.

Figure 6.1.

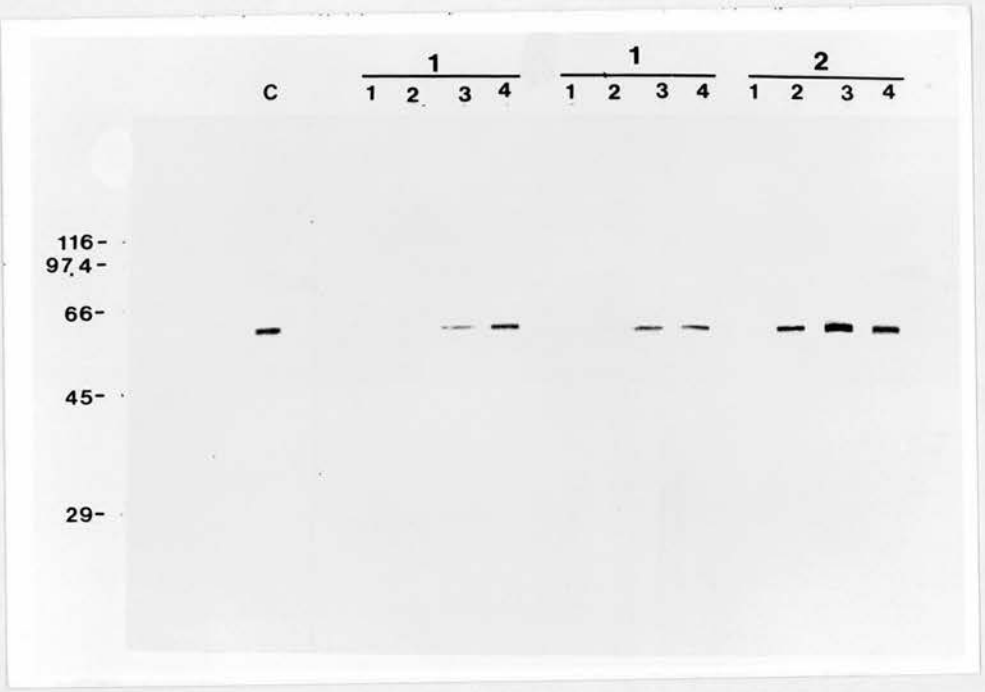
Serological responses of Groups 1 and 2 to LLO demonstrated by immunoblotting. (Serum dilution 1/100).

The significance of the superior figure: 1. Control Group 1 lambs; 2. Challenge Group 2 lambs. The lower figures indicate: C. positive control rabbit antiserum (1/500 dilution); lanes 1, day 0; lanes 2, day 21; lanes 3, day 35; lanes 4, day 49. The clinical cases of encephalomyelitis are identified by bracketed superscript letters.

Positions of molecular mass markers (kDa) shown.

Group 1 lambs dosed orally with PBS on days 0, 1, and 2 and injected subcutaneously with $2x10^{10}$ c.f.u. viable *L. monocytogenes* on day 24. Group 2 lambs dosed orally with $3x10^{10}$ c.f.u. viable *L. monocytogenes* over days 0, 1, and 2 and injected subcutaneously with $2x10^{10}$ c.f.u. viable *L. monocytogenes* on day 24.

Figure 6.1.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2
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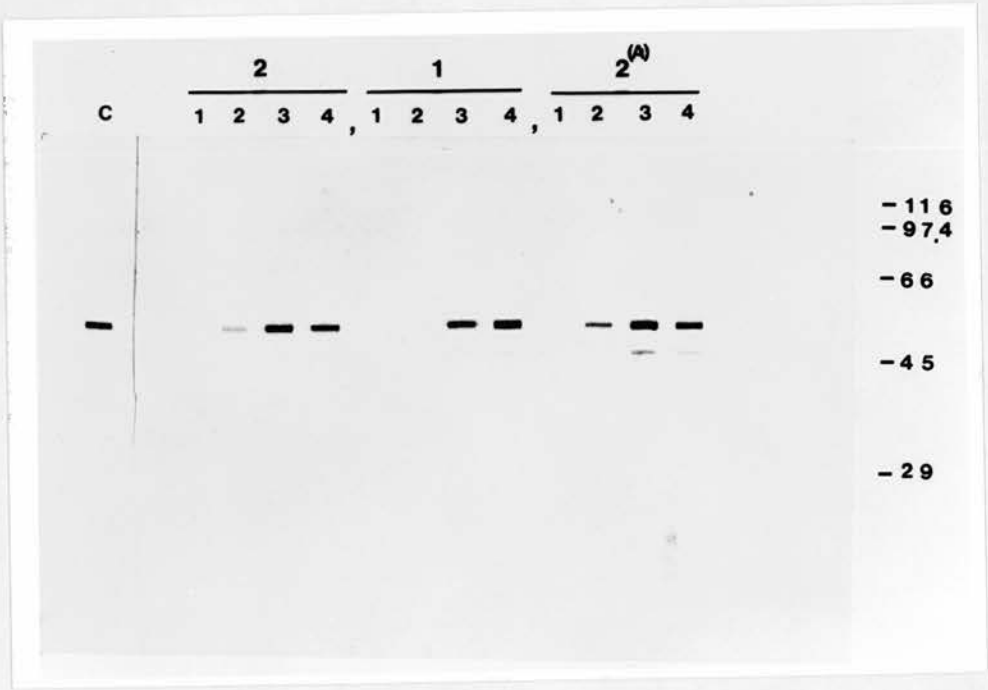
66-
45-
29-


C
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2(A)
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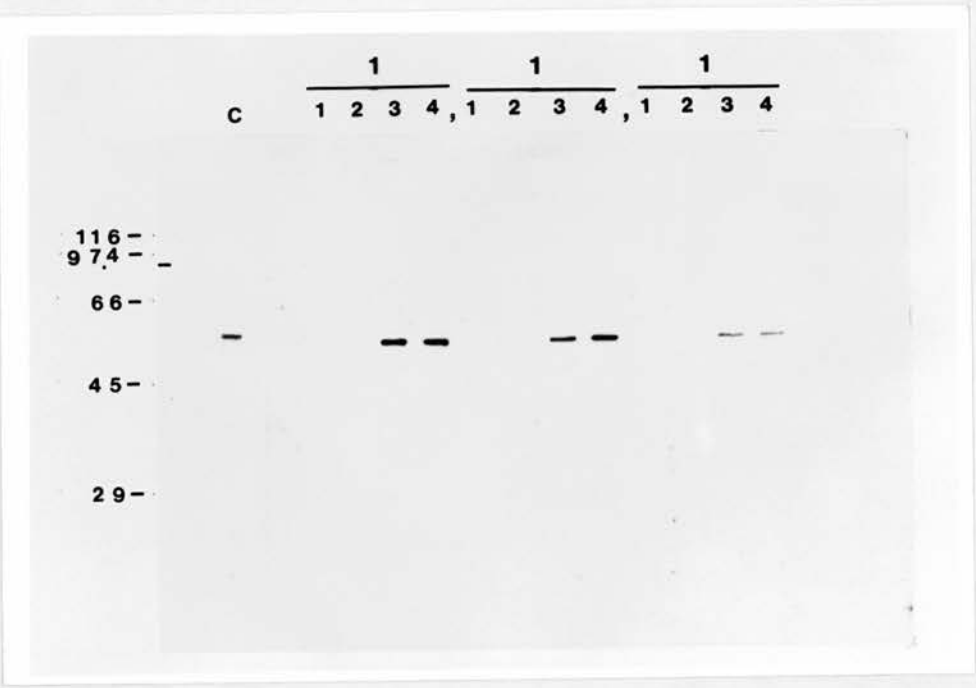


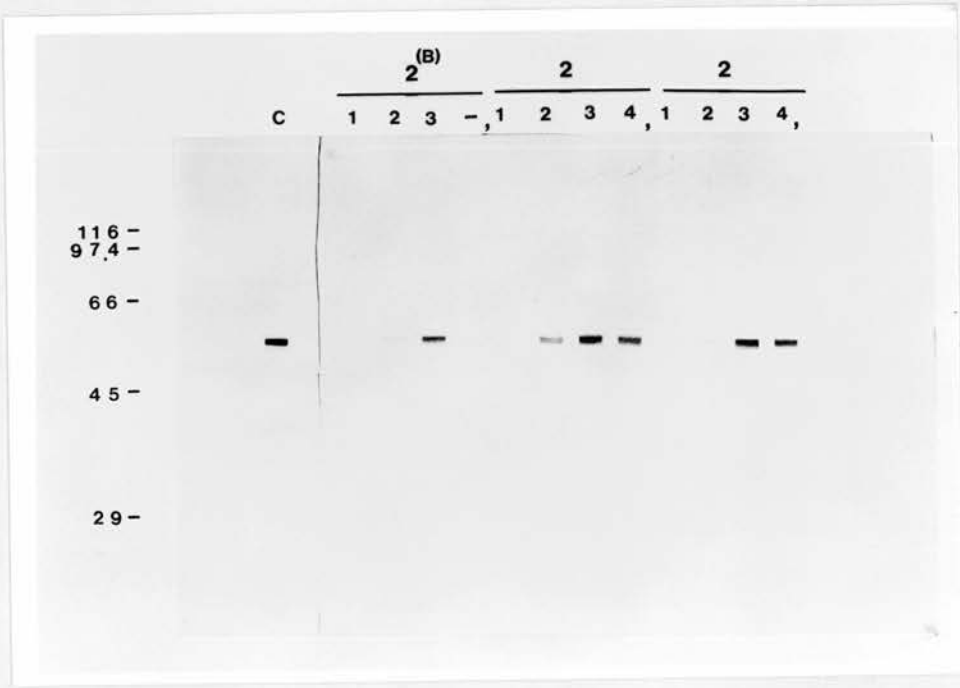


Figure 6.2.

Isotype of antibody recognizing LLO demonstrated by immunoblotting. (Above IgM response, below IgG_1 response). (Serum dilution 1/100).

The significance of the superior figure: 1. Control Group 1 lambs; 2. Challenge Group 2 lambs. The lower figures indicate: C. positive control rabbit antiserum (1/500 dilution); lanes 1, day 0; lanes 2, day 21; lanes 3, day 35; lanes 5, day 63.

Positions of molecular mass markers (kDa) shown.

Group 1 lambs dosed orally with PBS on days 0, 1, and 2 and injected subcutaneously with $2x10^{10}$ c.f.u. viable *L. monocytogenes* on day 24. Group 2 lambs dosed orally with $3x10^{10}$ c.f.u. viable *L. monocytogenes* over days 0, 1, and 2 and injected subcutaneously with $2x10^{10}$ c.f.u. viable *L. monocytogenes* on day 24.

Figure 6.2.

(Above, IgM and below, IgG_1 antibody responses).

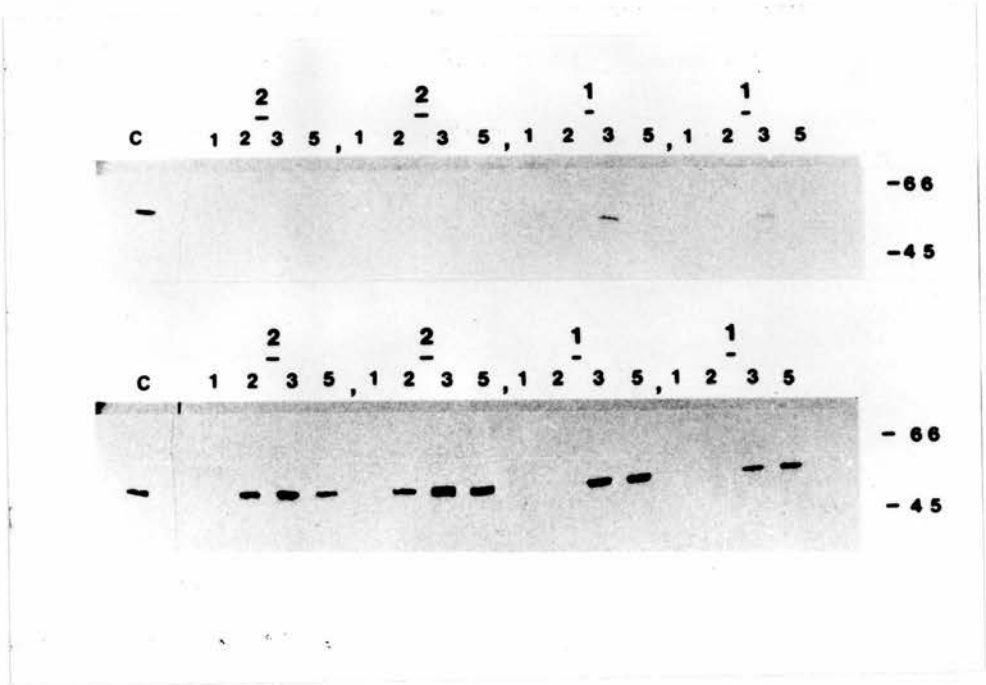


Figure 6.3.

Serological responses of Group 3 lambs to LLO demonstrated by immunoblotting. (Serum dilution 1/100).

The significance of the superior figure: 1. day 0; 2. day 14; 3. day 21. The lower figures indicate: C. positive control goat serum (1/100 dilution); lanes 1 to 6 are individual lambs.

Positions of molecular mass markers (kDa) shown.

Group 3 lambs dosed orally with $2x10^{10}$ c.f.u. heat-killed *L. monocytogenes* on days 0, 1 and 2.

Figure 6.3.

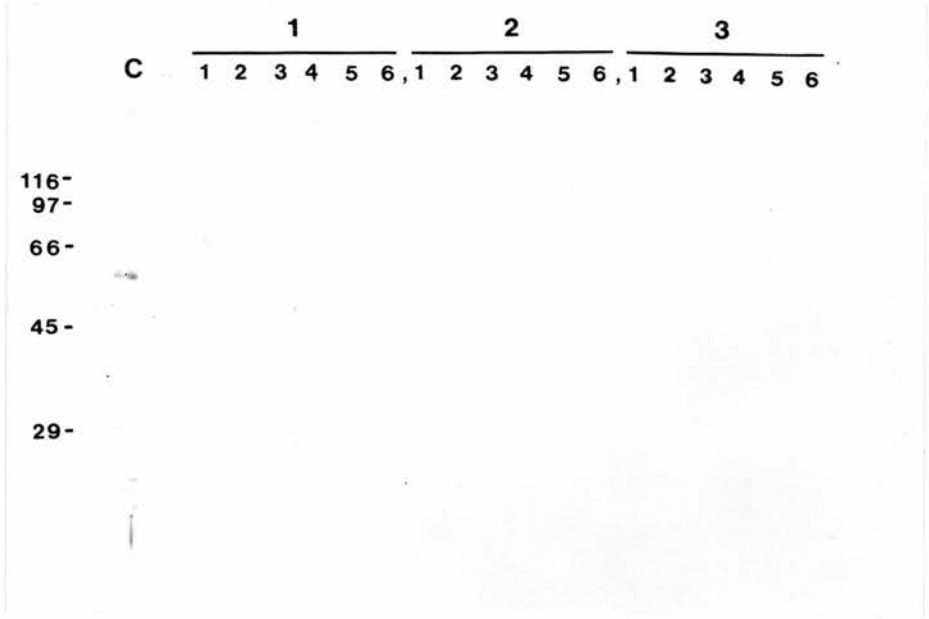
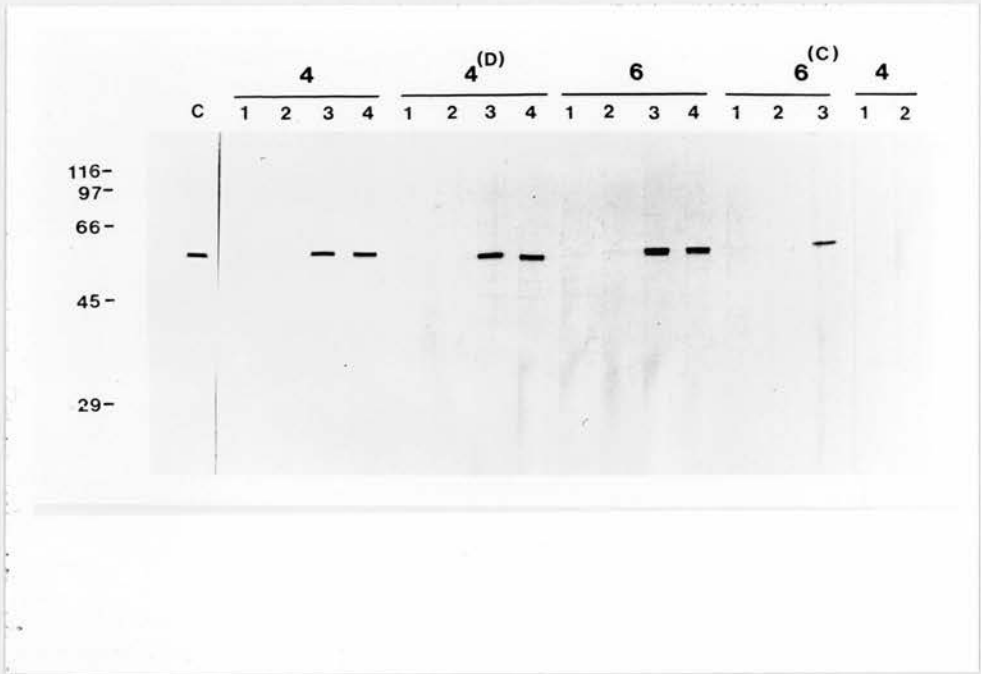


Figure 6.4.

Serological responses of Groups 4, 5 and 6 to LLO demonstrated by immunoblotting. (Serum dilution 1/100).

The significance of the superior figure: 4. Control Group 4 lambs, 5. Challenge Group 5 lambs, 6. Control Group 6 lambs. The lower figures indicate: C. positive control rabbit antiserum (dilution 1/500); lanes 1, day 0; lanes 2, day 21; lanes 3, day 35; lanes 4, day 49. Positions of molecular mass markers (kDa) shown. The clinical cases of encephalomyelitis are identified by bracketed superscript letters. Group 4 lambs dosed orally with PBS on days 0, 1 and 2, and injected subcutaneously with $8x10^8$ c.f.u. viable *L. monocytogenes* on day 21. Group 5 lambs dosed orally with in total $3.1x10^9$ c.f.u. viable *L. monocytogenes* over days 0, 1 and 2 and injected subcutaneously with $8x10^8$ c.f.u. viable *L. monocytogenes* on day 21. Group 6 lambs dosed orally with in total $4x10^9$ c.f.u. viable *L. innocua* over days 0, 1 and 2, and injected subcutaneously with $8x10^8$ c.f.u. viable *L. monocytogenes* on day 21.

Figure 6.4.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6(C)
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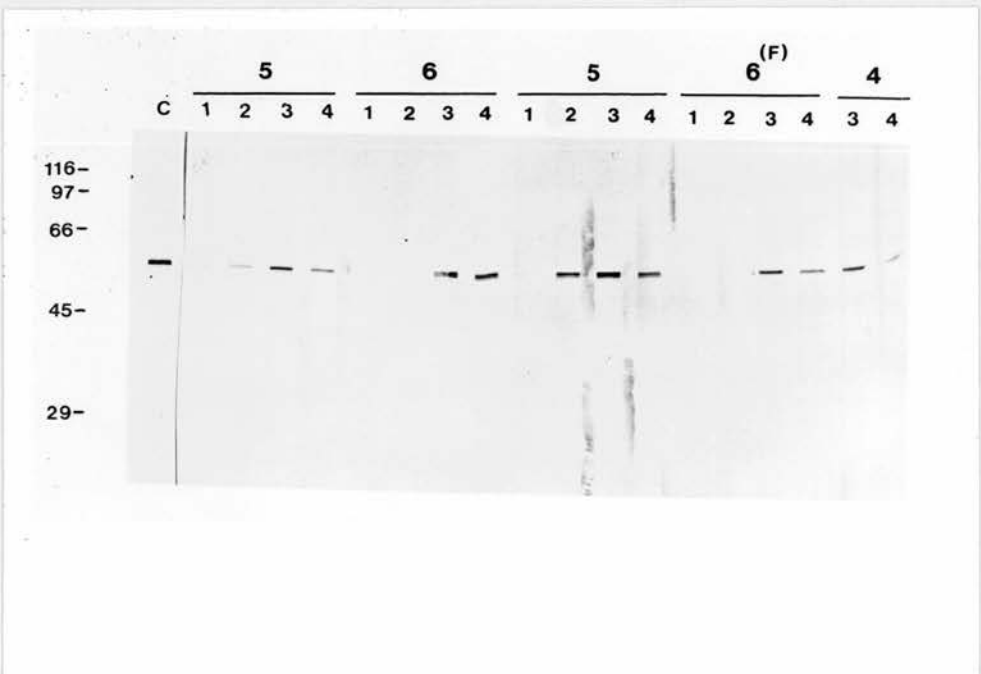
97-
66-
45-
29-


5
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5
6(F)
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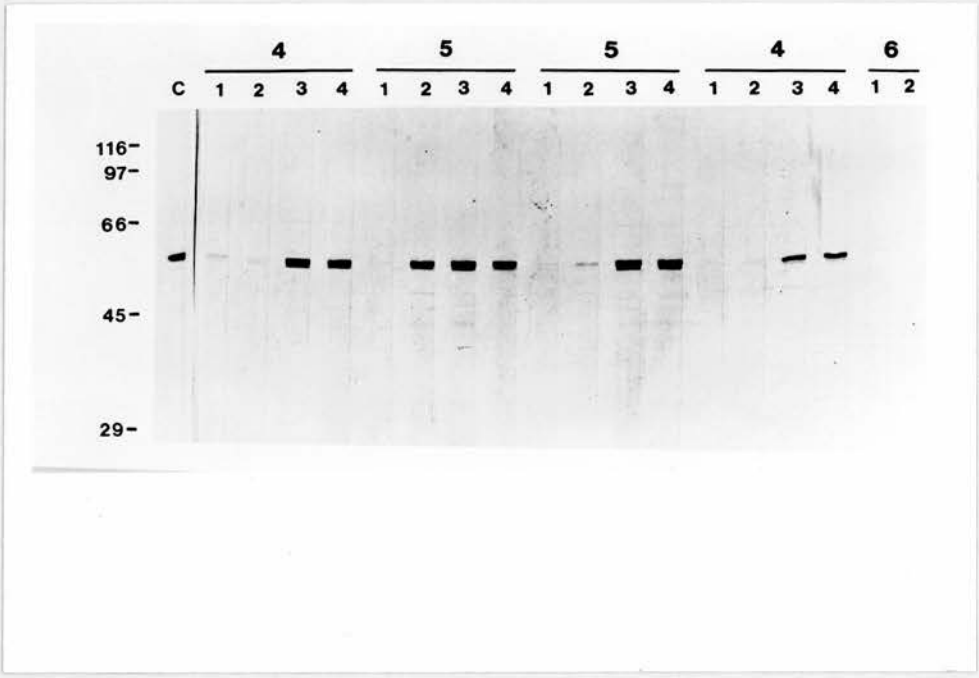
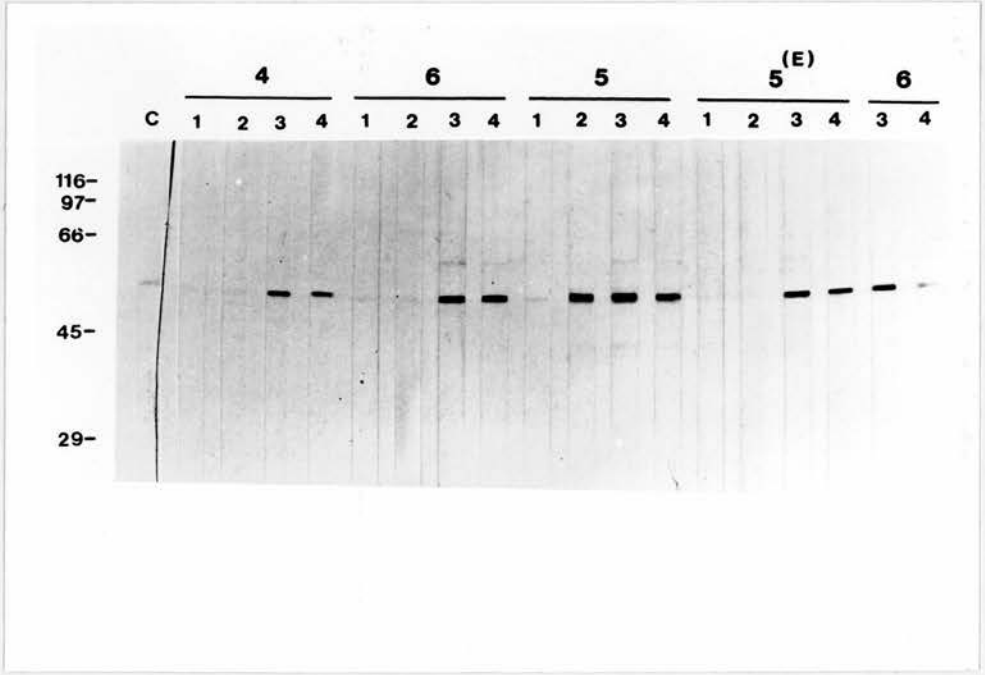
C 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 3 4
116-
97-
66-
45-
29-

Figure 6.4. (Cont'd).





6.2 Testing of sera by LLO ELISA

6.2.1 Testing of sera by indirect ELISA using purified LLO as antigen

Purified LLO (section 4.2) at a concentration of 250 ng ml^{-1} in 0.1M carbonate/bicarbonate buffer, pH 9.6, was coated on 96 well microtitre plates (Dynatech M129A) using the procedure described in section 4.3.3. The test sera were diluted 1/400 in serum buffer and the standard positive control was the convalescent goat serum (section 2.9.3) made up in eight two fold dilutions from 1/100 to 1/12800. The standard negative control serum, taken from a six month old conventional lamb, was diluted 1/400 in serum buffer. To the prepared plates 100 μl of standard or test sera were added to all wells except those in column 1 to which serum buffer alone was added. Each sample was tested in duplicate and plates were incubated at 37^{o}C for 60 min. The assays were then continued as described (section 2.8.3.1).

6.2.2 Calculation of quantitative anti-LLO antibody titres

The optical density was read at 492 nm wavelength in a multichannel recorder (Titertek Multiscan Flow Laboratories) interfaced to a BBC computer (Acorn Computer Ltd, Cambridge, UK) and Epson FX-80 printer using a Titertek Multiscan interface (Flow Laboratories) and a 3187 serial interface type 312B (Flow Laboratories).

The computer programme was designed to fit data from the standard curve to the following equation:

$$\log(y) = a + b(\log x)$$

where y = optical density of test serum, x = is the equivalent reciprocal value of test serum, a = is the log optical density at which the standard curve intercepts the y axis, b = is the slope of the standard. The titre of the standard serum was taken as being equal to the number of units of antibody contained therein at an optical density of 0.1 and the antibody titre in the test serum was derived from the following equation:

$$\text{titre of test serum} = \frac{\text{reciprocal of test serum dilution (i.e. 400)}}{\text{equivalent reciprocal of test serum (i.e. derived value } x)} \times \text{titre of standard}$$

6.2.3 Measurement of anti-LLO antibody levels in experimental sera

In the first experiment no antibody to purified LLO was detectable in any of the sera taken prior to challenge. However, following oral dosing with $3x10^{10}$ c.f.u. *L. monocytogenes* anti-LLO antibodies were detectable in five of the six lambs at 14 and 21 days. The control Group 1 lambs remained negative until after the subcutaneous challenges. On experimental day 35, all animals had detectable anti-LLO antibodies which were still present two weeks later (Table 6.6). ELISA titres rose to their maxima in Group 1 on day 49 and in Group 2 were maximal on day 35. Following oral dosing with heat-killed *L. monocytogenes* no anti-LLO antibodies were detectable in Group 3 lambs on days 7, 14, 21, or 28 (results not shown).

The anti-LLO responses of Groups 4, 5 and 6 are shown in Table 6.7. No recognition of LLO was demonstrable in any prebleed or in lambs of Groups 4 or 6 at day 21. In Group 5 anti-LLO antibodies were marked in four animals and undetectable in the other two lambs on day 21. After the subcutaneous injection of *L. monocytogenes* all the animals had detectable levels of anti-LLO antibodies which reached their maxima on day 35.

Table 6.6.

Serological responses of Groups 1 and 2 to LLO measured by indirect ELISA

Day	Group 1 (n=6)	Group 2 (n=6)
	(Antibody titre expressed as ELISA units ± SE).	
0	<400	<400
7	NT	NT
14	<400	4274±2486
21	<400	5160±2042
35	15430±1906	27512±3002
49	17332±2384	17236±2896 (n=5)

Test serum dilution 1/400. Group 1 dosed orally with PBS on days 0, 1 and 2, and injected subcutaneously with $2x10^{10}$ c.f.u. viable *L. monocytogenes* on day 24. Group 2 dosed orally with $3x10^{10}$ c.f.u. viable *L. monocytogenes* over days 0, 1 and 2, and injected subcutaneously with $2x10^{10}$ c.f.u. on day 24.

Table 6.7.

Serological responses of Groups 4, 5 and 6 to LLO measured by indirect ELISA

Day	Group 4 (n=6)	Group 5 (n=6)	Group 6 (n=6)
	(Antibody titre expressed as ELISA units ± SE).		
0	<400	<400	<400
7	NT	NT	NT
14	NT	NT	NT
21	<400	2063±844	<400
35	9850±3980	11684±2360	10359±3278
49	6802±3272	5125±1180	7014±1875 (n=5)
63	2824±1764 (n=5)	3127±889 (n=5)	4475±1472 (n=4)

Test serum dilution 1/400. Group 4 dosed orally with PBS on days 0, 1 and 2, and injected subcutaneously with $8x10^8$ c.f.u. viable *L. monocytogenes* on day 21. Group 5 dosed orally with $3.1x10^9$ c.f.u. viable *L. monocytogenes* over days 0, 1 and 2, and injected subcutaneously with $8x10^8$ c.f.u. on day 21. Group 6 dosed orally with $4x10^9$ c.f.u. viable *L. innocua* over days 0, 1 and 2, and injected subcutaneously with $8x10^8$ c.f.u. viable *L. monocytogenes* on day 21.

6.3 Humoral antibody responses of individual animals

To assess the sensitivity of the LLO ELISA and to permit a comparison of the serological responses of lambs which developed listeric encephalomyelitis the individual antibody responses are described. In all the lambs of Group 2 the percentage absorbance values measured by whole cell ELISA increased after oral dosing with viable *L. monocytogenes* and all developed anti-LLO antibodies detectable by immunoblotting. However one lamb, the poorest responder by the whole cell ELISA, was negative in SAT (result not shown) and had no detectable anti-LLO antibodies by ELISA (Table 6.8). By experimental day 35 all the assays revealed the lambs of both groups had seroconverted to *Listeria* somatic antigens and to LLO. In contrast, no individual seroconverted to somatic antigens or LLO following the oral dosing of Group 3 lambs with $6x10^{10}$ c.f.u. heat-killed *L. monocytogenes*.

After Group 5 lambs were orally dosed with viable *L. monocytogenes*, four of the animals were positive for anti-LLO antibodies by immunoblotting and ELISA and the percentage absorbance values by whole cell ELISA increased (Table 6.9). However, one animal in the group had no detectable anti-LLO response either by ELISA or immunoblotting and showed no increase in percentage absorbance values by whole cell ELISA. This lamb was

shown to be bacteraemic after the subcutaneous injection of *L. monocytogenes* and was assumed to have remained "uninfected" after oral dosing (section 5.3.4). One other lamb was negative for anti-LLO antibodies by ELISA and had a poor response by whole cell ELISA. The presence of anti-LLO antibodies was equivocal in immunoblots but for the calculation of sensitivity and specificity this immunoblotting result was assumed to be positive and therefore the LLO ELISA gave a false negative result.

The clinical cases of listeric encephalomyelitis (cases A and B) both had anti-LLO antibodies, detectable by ELISA and immunoblotting, after oral dosing and prior to the subcutaneous challenges. However, on day 21 the clinical cases of listeric encephalomyelitis in Groups 4, 5 and 6 (cases C, D, E and F) had no evidence of antibodies to listeric somatic antigens by whole cell ELISA or of anti-LLO antibodies by immunoblotting or ELISA. Each immunoassay showed these four lambs had seroconverted after the subcutaneous challenges but prior to the development of neurological symptoms. At no stage could the antibody responses of the encephalomyelitis cases be distinguished from the antibody responses of other lambs which remained clinically normal.

Table 6.8.

Comparison of Group 1 individual humoral antibody responses to somatic antigens measured by ELISA[a], and to LLO by indirect ELISA[b] and immunoblotting[c]

Animal	Day 0 Whole cell ELISA	Day 0 Anti-LLO response	Day 21 Whole cell ELISA	Day 21 Anti-LLO response	Day 35 Whole cell ELISA	Day 35 Anti-LLO response
1	5.2[a]	<400[b] (-)[c]	7.8[a]	<400[b] (-)[c]	91.1[a]	18936[b] (+)[c]
2	0.0	<400 (-)	4.3	<400 (-)	88.8	15722 (+)
3	3.6	<400 (-)	6.6	<400 (-)	63.2	17266 (+)
4	0.0	<400 (-)	3.9	<400 (-)	96.9	18502 (+)
5	4.8	<400 (-)	12.4	<400 (-)	85.3	15864 (+)
6	2.4	<400 (-)	7.8	<400 (-)	95.7	6284 (+)

Group 1 dosed orally with PBS on days 0, 1 and 2 and injected subcutaneously with $2x10^{10}$ c.f.u. *L. monocytogenes* on day 24.

Table 6.8. (Cont'd).

Comparison of Group 2 individual humoral antibody responses to somatic antigens measured by ELISA[a], and to LLO by indirect ELISA[b] and immunoblotting[c]

Animal	Day 0 Whole cell ELISA	Day 0 Anti-LLO response	Day 21 Whole cell ELISA	Day 21 Anti-LLO response	Day 35 Whole cell ELISA	Day 35 Anti-LLO response
1[A]	7.2[a]	<400[b] (-)[c]	72.9[a]	2170[b] (+)[c]	95.7[a]	27884[b] (+)[c]
2	8.0	<400 (-)	93.0	5994 (+)	93.4	27812 (+)
3	6.4	<400 (-)	55.0	5814 (+)	96.9	37586 (+)
4	4.0	<400 (-)	95.4	14248 (+)	98.8	33230 (+)
5	4.4	<400 (-)	34.5	<400 (+)	91.5	19796 (+)
6[B]	0.0	<400 (-)	73.6	2734 (+)	91.8	18768 (+)

Group 2 dosed orally with $1x10^{10}$ c.f.u. *L. monocytogenes* on days 0, 1 and 2 and injected subcutaneously with $2x10^{10}$ c.f.u. *L. monocytogenes* on day 24. The superscript capitals identify the first and second clinical encephalomyelitis cases.

Table 6.9.

Comparison of Group 4 individual humoral antibody responses to somatic antigens measured by ELISA[a], and to LLO by indirect ELISA[b] and immunoblotting[c]

Animal	Day 0 Whole cell ELISA	Day 0 Anti-LLO response	Day 21 Whole cell ELISA	Day 21 Anti-LLO response	Day 35 Whole cell ELISA	Day 35 Anti-LLO response
1	19.4[a]	<400[b] (-)[c]	16.7[a]	<400[b] (-)[c]	92.1[a]	6610[b] (+)[c]
2	12.3	<400 (-)	10.2	<400 (-)	93.6	3343 (+)
3	11.8	<400 (-)	17.2	<400 (-)	96.3	2056 (+)
4	17.3	<400 (-)	24.7	<400 (-)	92.5	14720 (+)
5[D]	16.0	<400 (-)	10.8	<400 (-)	90.2	27963 (+)
6	13.0	<400 (-)	13.5	<400 (-)	92.0	4408 (+)

Group 4 dosed orally with PBS on days 0, 1 and 2 and injected subcutaneously with $8x10^8$ c.f.u. *L. monocytogenes* on day 21. The superscript capital identifies the fourth clinical encephalomyelitis case.

Table 6.9. (Cont'd).

Comparison of Group 5 individual humoral antibody responses to somatic antigens measured by ELISA[a], and to LLO by indirect ELISA[b] and immunoblotting[c]

Animal	Day 0 Whole cell ELISA	Day 0 Anti-LLO response	Day 21 Whole cell ELISA	Day 21 Anti-LLO response	Day 35 Whole cell ELISA	Day 35 Anti-LLO response
1	26.4[a]	<400[b] (-)[c]	104.2[a]	4256[b] (+)[c]	97.9[a]	10801[b] (+)[c]
2	12.5	<400 (-)	33.6	<400 (±)	105.1	11053 (+)
3	13.7	<400 (-)	76.9	4256 (+)	90.1	22922 (+)
4[E]	12.7	<400 (-)	7.2	<400 (-)	86.9	5535 (+)
5	12.0	<400 (-)	91.7	507 (+)	90.8	10990 (+)
6	13.1	<400 (-)	97.4	3358 (+)	99.7	8800 (+)

Group 5 dosed orally with 3.1×10^9 c.f.u. *L. monocytogenes* over days 0, 1 and 2 and injected subcutaneously with 8×10^8 c.f.u. *L. monocytogenes* on day 21. The superscript capital identifies the fifth clinical encephalomyelitis case.

Table 6.9. (Cont'd).

Comparison of Group 6 individual humoral antibody responses to somatic antigens measured by ELISA[a], and to LLO by indirect ELISA[b] and immunoblotting[c]

Animal	Day 0 Whole cell ELISA	Day 0 Anti-LLO response	Day 21 Whole cell ELISA	Day 21 Anti-LLO response	Day 35 Whole cell ELISA	Day 35 Anti-LLO response
1	11.1[a]	<400[b] (-)[c]	8.9[a]	<400[b] (-)[c]	97.6[a]	8967[b] (+)[c]
2	14.4	<400 (-)	15.0	<400 (-)	96.7	4649 (+)
3	20.0	<400 (-)	17.5	<400 (-)	102.1	26447 (+)
4[C]	15.4	<400 (-)	12.0	<400 (-)	93.7	8967 (+)
5	15.4	<400 (-)	16.4	<400 (-)	87.4	4155 (+)
6[F]	14.3	<400 (-)	6.1	<400 (-)	84.6	8967 (+)

Group 6 dosed orally with $4x10^9$ c.f.u. *L. innocua* over days 0, 1 and 2 and injected subcutaneously with $8x10^8$ c.f.u. *L. monocytogenes* on day 21. The superscript capitals identify the third and sixth clinical encephalomyelitis cases.

6.4 Comparison of ELISA and immunoblotting for the detection of anti-LLO antibodies

The development of anti-LLO antibodies and their detection by immunoblotting was consistent with the presumed infection of animals following oral dosing with *L. monocytogenes*. The immunoblotting results were therefore used as the yardstick by which to measure the sensitivity of the LLO ELISA. The LLO ELISA produced one false negative result for a lamb in Group 2 and one assumed false negative result for a lamb in Group 5 (section 6.3). In comparison to immunoblotting the assay had a sensitivity of 82% and specificity of 100% for the detection of the putative oral infection by *L. monocytogenes*. The percentage agreement in the assays was 94% (Table 6.3). Both assays consistently detected anti-LLO antibodies in all the experimental animals after subcutaneous challenge with viable *L. monocytogenes*.

Table 6.3.

Comparison of ELISA and immunoblotting for the detection of anti-LLO antibodies following oral dosing with PBS or *Listeria spp.*

		Immunoblotting		
		positive	negative	total
LLO ELISA	positive	9[a]	0[b]	9
	negative	2[c]	25[d]	27
	total	11	25	36

ELISA sensitivity: $a/_{a+c}$ = 9/11 = 82%.

ELISA specificity: $d/_{b+d}$ = 25/25 = 100%.

% agreement: $a+d/_{a+b+c+d}$ = 34/36 = 94%.

Discussion.

Following oral dosing with viable *L. monocytogenes* antibody responses to *Listeria* somatic antigens by the SAT and whole cell ELISA were qualitatively similar. Though the percentage absorbance method for the expression of ELISA results produces values which are not linearly proportional to titre (de Savigny and Voller, 1980) the method is simple, rapid and relatively standard as the percentage is always derived from reference to a known positive antiserum (de Savigny and Voller, 1980). Both assays enabled the animals' serological responses to be monitored though no attempt was made to quantify the absolute antibody titres. The low SAT titres and low whole cell ELISA values found in the prebleeds of the conventional lambs agrees with many of the reports reviewed by Seeliger (1961) and suggests that the crude, ill-defined antigens used in both assays lack the specificity required for a useful immunoassay.

Seroconversion following oral dosing was a consequence of challenge by viable *L. monocytogenes* since no antibody response occurred when lambs were dosed with killed *L. monocytogenes*. Similarly after oral dosing with live *L. innocua* no antibody response to *L. monocytogenes* somatic antigens could be recognized. Since *L. monocytogenes* serovar 4b and *L. innocua* serovar 6a share the common

somatic antigens III and V it was assumed that infection by *L.innocua* would lead to an increase of ELISA percentage absorbance values using heat-killed *L. monocytogenes* serovar 4b as antigen. It is recognized that confirmation requires the measurement of serological responses after the experimental injection of animals with viable *L. innocua*.

The competitive sandwich ELISA for the detection of anti-LTA antibodies was only used in the initial challenge experiment as antibody responses were inconsistent, transient and weak and the assay was an unreliable indicator of listeric infection. In contrast LLO was identified by immunoblotting as a candidate antigen for a reliable serological assay as anti-LLO antibodies were consistently detected following both oral dosing and subcutaneous challenge with viable *L. monocytogenes*. In four lambs the predominant isotype of antibody response was IgG_1 which is in agreement with findings in man (Professor P. Berche, Institut Pasteur, Paris, personal communication). However, this failure to detect an IgM antibody response may be attributed to antibody decay associated with freeze/thawing of serum. Careful examination of further samples is merited to confirm the finding.

The purification of LLO (Chapter 4) allowed the development of a simple indirect ELISA for the detection of anti-LLO antibodies. The absorbance values were expressed as quantitative ELISA units based upon the method recommended by de Savigny and Voller (1980) in which absorbance values obtained at the test working dilution can be transformed by comparison to a standard curve. The inclusion on each plate of eight appropriate standard positive antiserum dilutions and eight replicates of a standard negative serum also allows for assessment of interplate variation associated with variations in incubation times and changes in batches of reagents (Hebert *et al.*, 1985; Lyaku *et al.*, 1990).

Using the anti-LLO ELISA no evidence for cross-reacting antibodies was found in the prebleeds of the conventional lambs despite their routine vaccination against clostridial diseases. Further no evidence of anti-LLO antibodies was detected in lambs after oral dosing with PBS, heat-killed *L. monocytogenes* or *L. innocua*. The subcutaneous injection of *L. monocytogenes* resulted in seroconversion to LLO and anti-LLO antibodies were consistently detected in all lambs both by immunoblotting and ELISA and maximal ELISA titres were reached 2 to 4 weeks after the subcutaneous challenges.

In assessing the accuracy of the anti-LLO assays the failure to isolate *L. monocytogenes* from the blood after oral dosing rendered imprecise the border between "infected" and "uninfected". However the clinical responses and the serological responses discerned by immunoblotting are evidence for "infection" having occurred in all Group 2 lambs after oral dosing with $3x10^{10}$ c.f.u. viable *L. monocytogenes*. One lamb, with the poorest whole cell ELISA response, though positive by immunoblotting was negative by the LLO ELISA and SAT. It was assumed for the calculation of sensitivity and specificity that this animal had been "infected" and that the LLO ELISA had produced a false negative result. In Group 5 the detection of anti-LLO antibodies in immunoblots was also taken to be an indication of whether animals had been "infected" after oral dosing with *L. monocytogenes* since the results were supported by the clinical responses following subcutaneous challenge. In this group one animal was "uninfected" after oral dosing and one false negative result was produced by the LLO ELISA. Overall, in comparison to immunoblotting the LLO ELISA had a sensitivity of 82% and a specificity of 100% for the determination of "infection" after oral challenge and the percentage agreement in the assays was 94%. After subcutaneous injection with viable *L. monocytogenes* the LLO ELISA and immunoblotting showed 100% agreement and an apparent sensitivity of 100%.

Field Studies

Chapter 7.

Detection of anti-listeriolysin O antibodies in field sera and the laboratory diagnosis of ovine listeriosis.

Introduction

In the previous chapter anti-listeriolysin O (anti-LLO) antibodies were detected in the sera of lambs after experimental listeric infection. However, it was impossible to categorically define if "infection" had occurred after the oral challenge and the sensitivity of the LLO ELISA could only be assessed upon the lambs' clinical and serological responses (section 6.4). Since the calculation of sensitivity should be based upon the true disease status of the animals and assessed independantly of the test under examination an alternative validation of the LLO ELISA was sought. This chapter includes the measurement of anti-LLO antibodies in sera from clearly defined field cases and an examination of the assay's sensitivity and specificity. The diagnosis of ovine listeric encephalitis by antigen detection is also briefly investigated.

7.1 Detection of anti-LLO antibodies in ovine serum by indirect ELISA

Serum samples were obtained from flocks which had not recorded cases of listeriosis, from those in which listeriosis was suspected upon clinical grounds and from individuals in which a clearly defined diagnosis was available. These sera were tested for the presence of anti-LLO antibodies using the previously described indirect ELISA (section 6.2). To avoid batch to batch variation all sera were tested in a single batch with reference sera from the experimental studies.

7.1.1 Anti-LLO antibody titres in the reference sheep sera

The reference sera were from the experimentally challenged lambs (section 5.1) and included: two sera taken from lambs three weeks after oral challenge with *L. monocytogenes*, one serum sample taken two weeks after the subcutaneous injection of a naive lamb with *L. monocytogenes* and another taken from a previously orally challenged lamb two weeks after *L. monocytogenes* was injected subcutaneously. The chosen sera were those closest to the means for each experimental group (section 6.2.3) and were tested in duplicate on each ELISA plate. For comparison the results are shown in Table 7.1.

Table 7.1.

Anti-LLO antibody titres in reference sera measured by indirect ELISA

		(Mean antibody titre expressed as ELISA units±SE).
Serum	1.	3916±93
	2.	4630±319
	3.	16729±827
	4.	28933±1975

Sera 1 and 2 taken three weeks after the oral dosing of naive lambs with *L. monocytogenes*. Serum 3 taken two weeks after the subcutaneous injection of a lamb with *L. monocytogenes*. Serum 4 taken from a previously orally challenged lamb two weeks after the subcutaneous injection of *L. monocytogenes*. The mean titre was calculated from four sets of results.

7.1.2 The prevalence of anti-LLO antibodies in five sheep flocks

Twenty five serum samples were obtained at random from adult ewes of flock 1 which used hay for feeding and had not recorded cases of listeriosis in the previous five years. Serum samples were also received from 24 sheep amongst 500 ewes (flock 2) where 14 ewes had developed severe diarrhoea (group A) six weeks after introduction to silage feeding and five days later eight abortions had occurred (group B). *L. monocytogenes* serovar 1/2a was isolated on the direct culture of fetal stomach contents and placentas of two abortion specimens examined. The majority of the ewes showed no clinical signs of infection (group C) and group D animals were only fed silage for three weeks prior to the outbreak. Blood samples were collected from six ewes in each of these four groups two days after the abortions occurred.

Flock 3 was a flock of 50 ewes in which animals became ill two days after the introduction of silage feeding. In total 14 ewes died, three abortions occurred and many of the ewes appeared ill. *L. monocytogenes* was isolated from the liver, kidney and gut of adult animals submitted for postmortem examination but isolates were not serotyped. Five serum samples were collected eleven days after the introduction of silage feeding though the

clinical histories for the individual animals were unavailable. Nineteen serum samples from clinically healthy, adult ewes in contact with confirmed cases of listeric encephalitis were obtained from flocks 4 and 5. At the time of sampling flock 4 had been feeding silage for four weeks and flock 5 for 11 weeks.

The 25 sera from the hay fed flock 1 were negative for anti-LLO antibodies. In flock 2 all animals in group A and five animals in group B had anti-LLO antibody titres similar to those seen following experimental challenge with *L. monocytogenes*. In group C, which had shown no clinical signs of infection, antibodies to LLO were demonstrated in three animals. For the animals in group D, which had been fed silage for only a short period, one animal had a low anti-LLO antibody titre (Table 7.2).

In four of the five ewes in flock 3 anti-LLO antibody titres were consistent with listeric infection (Table 7.3). Antibody values comparable with those seen after experimental oral challenge were present in 53% and 50% of the samples from flocks 4 and 5 (Tables 7.4 and 7.5).

Table 7.2.

Anti-LLO antibody titres in flock 2 measured by indirect ELISA

	Group A	Group B	Group C	Group D
	(Antibody titre expressed as ELISA units).			
Animal 1.	4838	<400	<400	<400
2.	4131	598	<400	<400
3.	19646	7895	2907	<400
4.	1165	26963	12716	895
5.	20238	5180	<400	<400
6.	6101	30870	32806	<400

Group A ewes had severe diarrhoea seven days before sampling. Group B ewes had aborted two days before sampling. Group C ewes were silage-fed but remained clinically normal. Group D ewes had only been fed silage for three weeks.

Table 7.3.

Anti-LLO antibody titres in flock 3 measured by indirect ELISA

	(Antibody titre expressed as ELISA units).
Animal 1.	9510
2.	34868
3.	<400
4.	10604
5.	24984

50 ewe silage fed flock. 14 animals died of listeric septicaemia, three aborted and most ewes appeared ill.

Table 7.4.

Anti-LLO antibody titres in flock 4 measured by indirect ELISA

	(Antibody titre expressed as ELISA units).
Animal 1.	10621
2.	520
3.	<400
4.	10946
5.	2799
6.	<400
7.	<400
8.	599
9.	<400
10.	<400
11.	1191
12.	<400
13.	<400
14.	1434
15.	3217

Fifteen clinically normal ewes in contact with confirmed cases of listeric encephalitis.

Table 7.5.

Anti-LLO antibody titres in flock 5 measured by indirect ELISA

	(Antibody titre expressed as ELISA units).
Animal 1.	3022
2.	845
3.	<400
4.	<400

Four clinically normal ewes in contact with confirmed cases of listeric encephalitis.

7.1.3 The prevalence of anti-LLO antibodies in proven cases of listeriosis

Serum samples were taken immediately prior to death from 16 sheep with clinical symptoms of listeric encephalitis and from a single ewe immediately after abortion. These sera together with a convalescent serum sample taken three weeks later from the same aborted ewe were tested for the presence of anti-LLO antibodies by indirect ELISA. All cases of listeric encephalitis were confirmed by histological examination of the brains and the listeric abortion was diagnosed by isolation of the organism from the fetal stomach contents.

Seven of the encephalitis cases were negative for anti-LLO antibodies (Table 7.6) and eight of the remaining nine had antibody titres similar to those seen after experimental oral challenge. Only one animal had an anti-LLO titre comparable to those detected after subcutaneous injection with the organism. The aborted ewe had a high titre which was considerably lower three weeks later (Table 7.7).

Table 7.6.

Anti-LLO antibody titres of confirmed listeric encephalitis cases measured by indirect ELISA

Animal	Age	(Antibody titre expressed) (as ELISA units)	Serotype of isolate
1.	11 months	501	not serotyped
2.	2 years	4254	not serotyped
3.	2 years	4556	no isolate
4.	11 months	3165	no isolate
5.	15 months	<400	no isolate
6.	5 years	2034	*L. mono* 1/2a
7.	Adult	2530	*L. mono* 1/2a
8.	6 weeks	<400	not serotyped
9.	1 year	12760	*L. mono* 1/2a
10.	6 weeks	<400	*L. mono* 1/2a
11.	6 weeks	<400	no isolate
12.	3 years	610	*L. mono* 1/2a
13.	7 years	<400	*L. mono* 1/2a
14.	1 year	<400	*L. mono* 1/2a
15.	5 years	1165	*L. mono* 4b
16.	8 weeks	<400	*L. mono* 1/2a

Table 7.7.

Anti-LLO antibody titre of a listeric abortion case measured by indirect ELISA

(Antibody titre expressed as ELISA units).	
Acute sample	20222
Convalescent sample	3243

7.1.4 The prevalence of anti-LLO antibodies in normal sheep and those suffering diseases other than listeriosis

Serum samples from six clinically normal sheep and six sheep suffering diseases other than listeriosis were all negative for anti-LLO antibodies (Table 7.8). The clinical diagnoses are listed in the table.

Table 7.8.

Anti-LLO antibody titres of clinically normal sheep and those suffering diseases other than listeriosis measured by indirect ELISA

Animal	Age	(Antibody titre expressed) (as ELISA units)	Diagnosis
1.	5 years	<400	normal
2.	5 years	<400	normal
3.	5 years	<400	normal
4.	Adult	<400	normal
5.	Adult	<400	normal
6.	Adult	<400	normal
7.	6 years	<400	hypocalcaemia
8.	2 years	<400	parasitic - gastroenteritis
9.	9 months	<400	acidosis
10.	Adult	<400	scrapie
11.	2 years	<400	cerebrocortical -necrosis
12.	1 week	<400	streptococcal -arthritis

7.2 Testing of field sera for recognition of LLO and SLO by immunoblotting

After SDS-PAGE in 10% polyacrylamide resolving gels, samples of 5 μg purified LLO (section 4.2) or 5 μg SLO (Sigma, Poole, England) were electrophoretically transferred to Immobilon-P membranes and 17 of the sera from flock 2, diluted 1/100 in serum diluent, were tested for recognition of LLO and SLO by immunoblotting (section 2.8.10). A horse anti-SLO serum (Wellcome Diagnostics, Dartford, England) and a hyperimmune rabbit anti-LLO serum (section 2.9.4) diluted to 1/400 and 1/500 respectively were used as positive controls in the appropriate immunoblots. Skimmed milk powder (5% w/v) was used in place of horse serum in the blocking buffer and 1% (w/v) skimmed milk powder in the serum diluent. The anti-sheep and anti-rabbit horseradish peroxidase conjugates were obtained from SAPU and the anti-horse horseradish peroxidase conjugate from Sigma. The conjugates were used at dilutions of 1/200 in serum diluent and the assays developed as previously described (section 2.8.10).

Anti-LLO antibodies were demonstrated in the field sera by immunoblotting (Figure 7.1) and results correlated with those obtained by indirect ELISA (Table 7.2). A weak reaction to SLO was apparent in a single serum sample (Figure 7.1).

Figure 7.1.

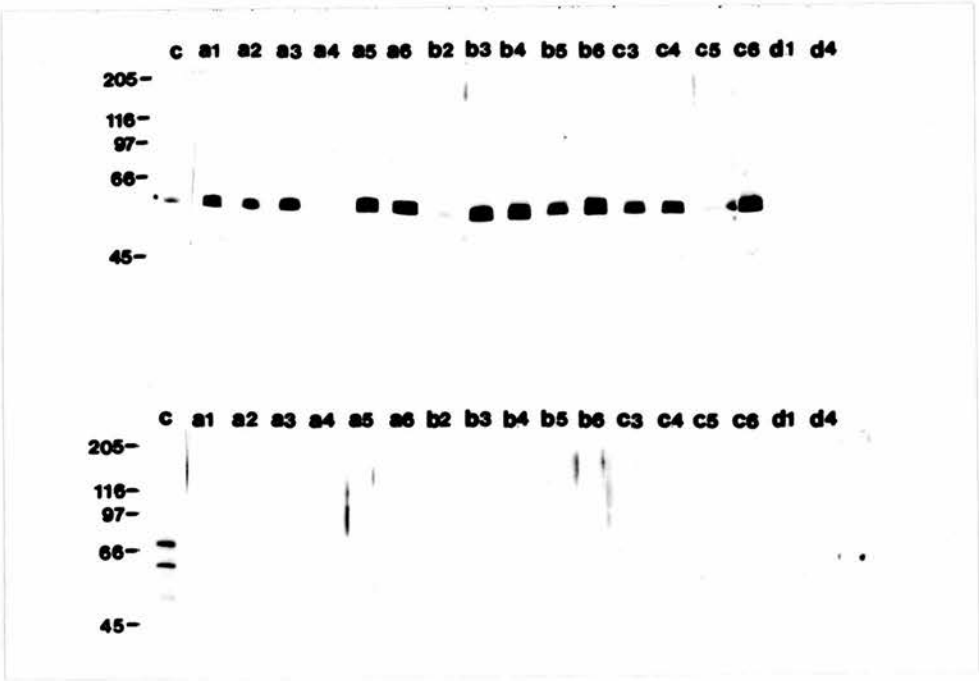
Examination of field sera for recognition of LLO and SLO by immunoblotting.

C indicates lanes probed with positive control antisera. Responses to LLO (above) and SLO (below) by 17 field serum samples are visible in the marked lanes. Group A ewes had severe diarrhoea seven days before sampling. Group B ewes had aborted two days before sampling. Group C ewes were silage-fed but remained clinically normal. Group D ewes had only been fed silage for three weeks. The sera specifically recognised purified LLO and the results of immunoblotting correlated with the LLO ELISA titres (Table 7.2).

Position of molecular mass markers (kDa) are indicated as numbers.

Figure 7.1.

(Above, anti-LLO and below, anti-SLO responses).



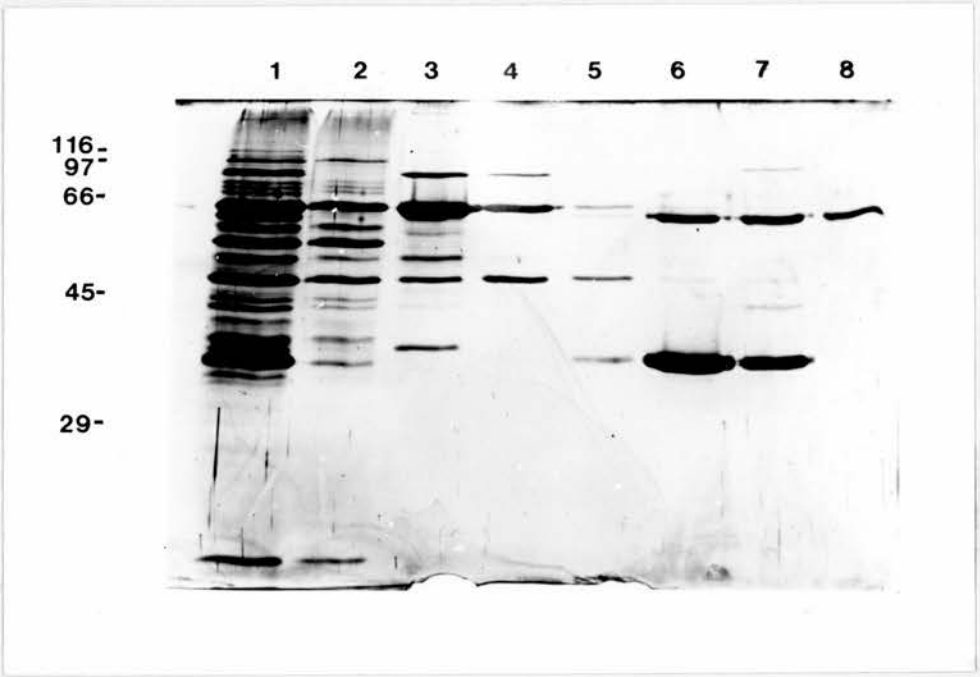
7.3 Purification of LLO from *L. monocytogenes* serovar 1/2a and testing of field sera for serovar specific anti-LLO antibodies

7.3.1 Purification of LLO from *L. monocytogenes* serovar 1/2a (strain L72)

Haemolysin was obtained from *L. monocytogenes* serovar 1/2a after growth of the bacterium in dialysis sac culture and SP-cation exchange chromatography of the culture supernatant fluid as described in section 4.2. The elution profile and yield of haemolysin were similar to those described in chapter 4. However, in SDS-PAGE the column fraction with peak haemolytic activity (chromatography fraction 13) was shown by silver staining to contain two proteins with molecular masses of 58 kDa and 35 kDa (Figure 7.2). In immunoblot the 58 kDa protein from the serovar 1/2a strain was recognized by the convalescent goat serum raised against a serovar 4b strain of *L. monocytogenes* but there was no apparent recognition of the 35 kDa protein (Figure 7.3). For clarity in the subsequent sections the 58 kDa protein is referred to as LLO though it must be acknowledged that formal identification of the protein was not undertaken.

Figure 7.2.

Silver staining of *L. monocytogenes* serovar 1/2a dialysis sac culture supernatant fluid and SP-cation exchange fractions in SDS-PAGE.

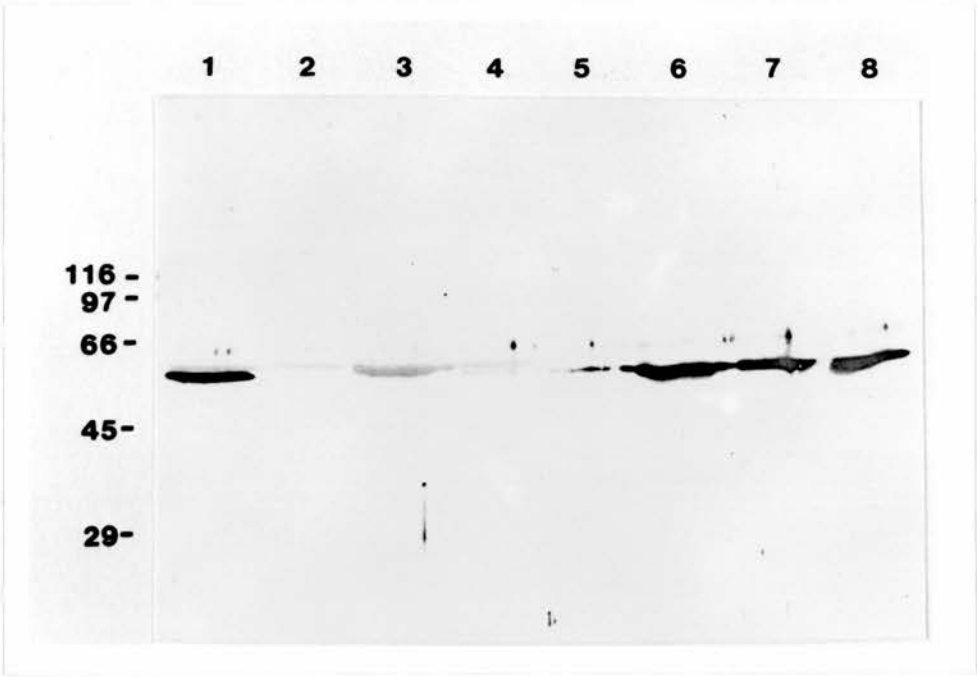


Lane 1, *L. monocytogenes* serovar 1/2a 10X dialysis sac supernatant fluid; lane 2, SP-cation exchange column fall through; lane 3, fraction 10 collected from SP-cation exchange column; lane 4, fraction 11; lane 5, fraction 12; lane 6, fraction 13; lane 7, fraction 14; lane 8, purified LLO from *L. monocytogenes* serovar 4b (0.5 μg protein).

Position of molecular mass markers (kDa) shown.

Figure 7.3.

Immunoblot of *L. monocytogenes* serovar 1/2a dialysis sac culture supernatant fluid and SP-cation exchange fractions probed with the convalescent goat serum.



Lane 1, *L. monocytogenes* serovar 1/2a 10X dialysis sac supernatant fluid; lane 2, SP-cation exchange column fall through; lane 3, fraction 10 collected from SP-cation exchange column; lane 4, fraction 11; lane 5, fraction 12; lane 6, fraction 13; lane 7, fraction 14; lane 8, purified LLO from *L. monocytogenes* serovar 4b (0.5 μg protein). Convalescent goat serum used at a dilution of 1/200.

Position of molecular mass markers (kDa) shown.

The major protein in column fraction 10 had a molecular mass of 60 kDa whereas this protein from the serovar 4b strain had an apparent molecular mass of 58 kDa, identical to that of LLO (Figure 4.2). In contrast to the result shown in figure 4.3 there was weak recognition of the 60 kDa protein which is attributed to use of the convalescent goat serum at a 1/200 dilution rather than 1/500.

7.3.2 Testing of field sera for serological recognition of *L. monocytogenes* serovar 1/2a LLO by immunoblotting

The SP-cation exchange column fraction with peak haemolytic activity (approximately 5 μg LLO) was prepared for SDS-PAGE as described (section 2.8.7.4). After electrophoresis in a 10% polyacrylamide resolving gel proteins were electrophoretically transferred to Immobilon P membranes and seventeen of the sera from flock 2, diluted 1/100 in serum diluent, were tested for recognition of LLO by immunoblotting. The convalescent goat serum was used as positive control. Anti-LLO antibodies were demonstrated in the field sera (Figure 7.4) and the results showed a similarity to those obtained by indirect ELISA and immunoblotting using as antigen LLO purified from *L. monocytogenes* serovar 4b. The 35 kDa contaminant protein transferred to the membrane and was visualised through Ponceau red staining. However, the field sera showed no immunological recognition of this 35 kDa protein.

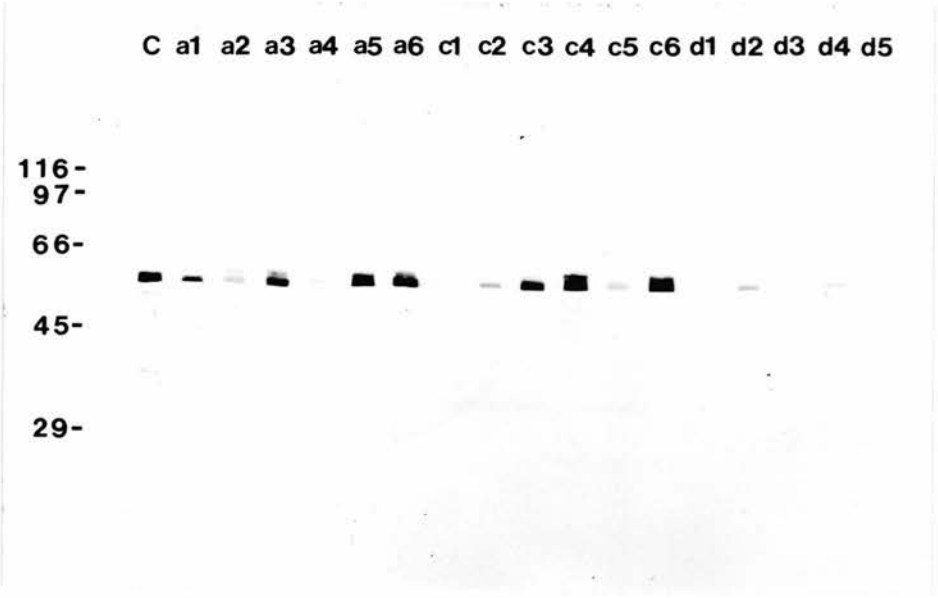
Figure 7.4.

Testing of field sera for recognition of *L. monocytogenes* serovar 1/2a LLO by immunoblotting.

C indicates lane probed with positive control convalescent goat serum. Responses to LLO by 17 field serum samples are visible in the marked lanes. Group A ewes had severe diarrhoea seven days before sampling. Group C ewes were silage-fed but remained clinically normal. Group D ewes had only been fed silage for three weeks. The sera recognised LLO derived from the serovar 1/2a strain and results were comparable with those obtained using LLO purified from a serovar 4b strain (Table 7.2).

Position of molecular mass markers (kDa) are indicated as numbers.

Figure 7.4.



7.3.3 Serological recognition of LLO from *L. monocytogenes* serovars 1/2a and 4b determined by indirect ELISA

The 24 field sera from flock 2 and sera from the 30 individuals described in sections 7.1.3 and 7.1.4 were tested by indirect ELISA for serological recognition of LLO purified from *L. monocytogenes* serovars 1/2a and 4b. The ELISA test procedure for the detection of antibodies to LLO purified from *L. monocytogenes* serovar 4b was as described in section 6.2. The test sera were diluted 1/400 in serum buffer and the standard positive control was the convalescent goat serum (section 2.9.3) made up in eight two fold dilutions from 1/100 to 1/12800. The standard negative control serum, taken from a six month old conventional lamb, was diluted 1/400 in serum buffer. The ELISA procedure for detection of antibodies to the *L. monocytogenes* serovar 1/2a LLO used the same serum dilutions and exactly the same procedure. ELISA plates were prepared by diluting the serovar 1/2a haemolysin preparation (chromatography fraction 13, section 7.3.1) in 0.1M carbonate/bicarbonate buffer, pH 9.6 to approximately 250 ng protein ml^{-1} and coating each well of 96 well microtitre plates (Dynatech M129A) with 100 μl volumes. Plates were left sealed at $4^{o}C$ overnight before continuation of the assay as described.

The optical densities obtained by ELISA using the LLO preparations from *L. monocytogenes* serovars 1/2a and 4b were arranged into the following categories: <0.1, 0.10 to 0.50, 0.51 to 1.0 and >1.0. Comparison of the results shows that the 24 sera from flock 2, the two sera from the listeric abortion case and the 12 sera from clinically normal sheep and those suffering diseases other than listeriosis fall into the same categories with both preparations. Though the results for the confirmed cases of listeric encephalitis differ there is no clear evidence for a serovar specific recognition of the serovar 1/2a LLO (Table 7.9). (The actual optical densities are given in Appendix 2).

Table 7.9.

Comparison of the optical densities of field sera obtained by indirect ELISA using LLO purified from *L. monocytogenes* serovars 1/2a or 4b

	Number of sera from flock 2 in each optical density category using as ELISA antigen:	
	serovar 1/2a	serovar 4b
O.D.	LLO	LLO
<0.10	9	9
0.10-0.50	7	7
0.51-1.00	4	4
>1.00	4	4

Table 7.9. (Cont'd).

Number of sera from confirmed listeric encephalitis cases in each optical density category using as ELISA antigen:

	serovar 1/2a	serovar 4b
O.D.	LLO	LLO
<0.10	4	8
0.10-0.50	11	8
0.51-1.00	1	0
>1.00	0	0

Number of sera from a listeric abortion case in each optical density category using as ELISA antigen:

	serovar 1/2a	serovar 4b
O.D.	LLO	LLO
0.10-0.50	1	1
0.51-1.00	1	1

Table 7.9. (Cont'd).

Number of sera from clinically normal sheep and those suffering diseases other than listeriosis in each optical density category using as ELISA antigen:

	serovar 1/2a	serovar 4b
O.D.	LLO	LLO
<0.10	11	11
0.10-0.50	1	1
0.51-1.00	0	0
>1.00	0	0

7.4 Attempts to detect LLO in cerebrospinal fluid samples by immunoblotting

Cerebrospinal fluid samples collected from five confirmed cases of listeric encephalitis and from one lamb suffering streptococcal arthritis were diluted 1:1 in double strength SDS-PAGE sample buffer and boiled for 3 min. Individual samples were applied in 20 μl volumes to two SDS-PAGE gels alongside 0.125, 0.25 and 0.5 μg of purified LLO (section 4.2). Following electrophoresis through 10% polyacrylamide resolving gels proteins were electrophoretically transferred to Immobilon-P membranes in a wet blot tank (section 2.8.10). After blocking of free binding sites the membranes were incubated in 1/500 dilutions of the hyperimmune anti-LLO rabbit serum produced in Chapter 4 (section 4.4). After extensive washing one membrane was incubated in a 1/200 dilution of an anti-rabbit horseradish peroxidase conjugate (SAPU) and the second membrane in a 1/500 dilution of a goat biotinylated anti-rabbit serum (Sigma). The assay of the first membrane was completed as previously described (section 2.8.10) whilst after thorough washing the second membrane was incubated in a 1/500 dilution of extravidin peroxidase (Sigma) before development in the same manner. In both immunoblots the control antiserum detected all three dilutions of antigen but LLO could not be detected in any of the CSF samples (Figure 7.5).

Figure 7.5.

Attempts to detect LLO in cerebrospinal fluid samples by immunoblotting.

(Above anti-rabbit horseradish peroxidase conjugate, below extravidin peroxidase).

Lane 1, purified LLO (0.5 μg protein); lane 2, LLO (0.25 μg protein); lane 3, LLO (0.125 μg protein); lane 4, CSF sample from a lamb suffering streptococcal arthritis; lanes 5 to 9, CSF samples from confirmed listeric encephalitis cases.

Figure 7.5. (anti-rabbit horseradish peroxidase conjugate).

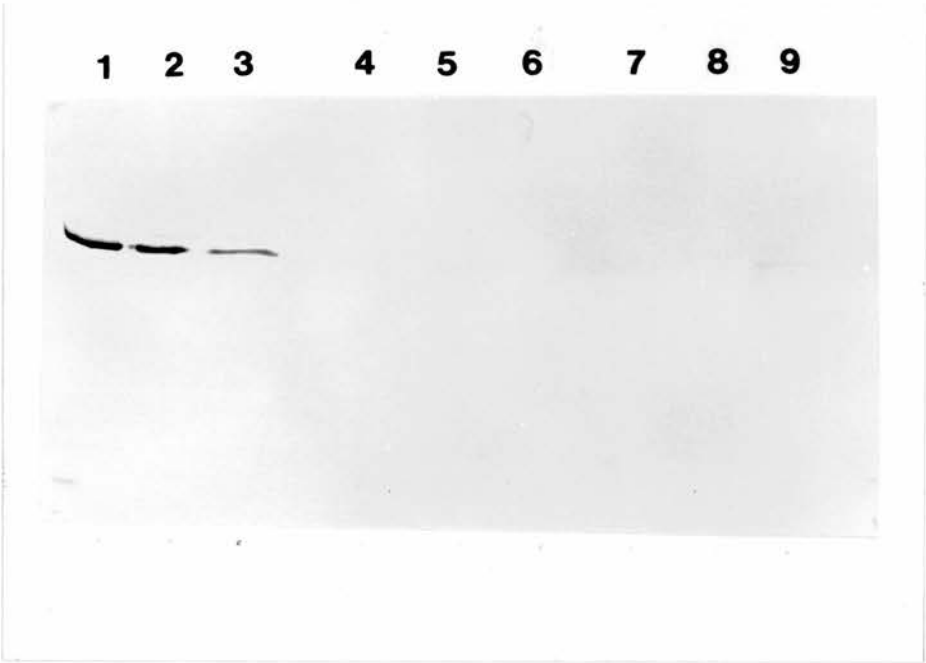


Figure 7.5. (extravidin peroxidase conjugate).

Discussion

Anti-LLO antibody titres comparable with those found after subcutaneous injection with *L. monocytogenes* were present in groups A and B of flock 2 and in four of the five ewes of flock 3. Although individual histories were unavailable these ewes were suspected of having suffered clinical listeriosis and the descriptions of the field outbreaks of disease were consistent with a previous report of listeric septicaemia and abortion (Low and Renton, 1985). Anti-LLO antibodies were detected in three ewes from group C of flock 2 and in 53% and 50% of ewes in flocks 4 and 5. Titres were comparable with those found after experimental infection and the results suggest that these animals were exposed to infection but remained clinically normal as is consistent with the experimental results described in Chapter 5. Since no anti-LLO antibodies were detected in flock 1 these subclinical listeric infections are presumably associated with silage feeding. The finding supports the report of Osebold and Aalund (1968) in which serum agglutination tests with 2-mercaptoethanol reduced sera revealed agglutinating titres to *L. monocytogenes* in 21% and 53% of clinically healthy ewes from two flocks experiencing cases of listeric encephalitis.

Despite routine vaccination against clostridial disease cross reactive antibodies to LLO were uncommon in ovine field sera. Only a low anti-LLO titre was detected in a single ewe from group D of flock 2 and no anti-LLO antibodies were found in the 25 ewes in flock 1 or the twelve ewes described in section 7.1.4. The specificity of the LLO ELISA was also studied by examining the reaction of field sera with purified LLO and SLO in immunoblots. The LLO ELISA titres for the field sera correlated with the anti-LLO immunoblotting results. However no anti-SLO antibodies were detectable, except for a single weak response, and the result is evidence for specific antigenic epitopes in LLO as reported by Nato *et al.* (1991). It appears that these epitopes are recognized during the course of natural listeric infections and the LLO ELISA is therefore detecting specific anti-LLO antibodies.

There have been few studies of the serological responses of animals suffering listeric encephalitis. Peel (1987) could not detect anti-flagellin antibodies in 16 cases but since *Listeria* do not synthesize flagellin at 37°C (Peel *et al.*, 1988a) the antigen was inappropriate for serodiagnosis and the assay has since been shown to be incapable of detecting a serological response in experimentally infected animals (Low and Donachie, 1991). Antibody titres against whole cell antigens, measured by ELISA, were similar to titres found in normal ewes (Peel, 1987). Similarly, Loken and

Gronstol (1982) could not distinguish haemagglutinating antibody titres in six caprine cases of listeric encephalitis from those in healthy goats of the same herd. In the current work, seven out of 16 confirmed cases of listeric encephalitis had no detectable antibodies to LLO and the majority had low titres comparable to those seen after experimental oral challenge. It is interesting to note that five of the seven seronegative animals were under eight weeks of age and one was a seven year old ewe from a flock which did not practise silage feeding. These results imply that the anti-LLO titres found in the encephalitis cases reflect those demonstrated in the clinically normal ewes of flocks 4 and 5 and in group C of flock 2.

Since the majority of cases of listeric encephalitis were due to *L. monocytogenes* serovar 1/2a it was possible the negative results were due to a failure of anti-LLO antibodies to bind with LLO purified from a serovar 4b strain. It was therefore decided to examine these sera for the recognition of LLO obtained from a serovar 1/2a strain of *L. monocytogenes*. Although a haemolysin was purified from the serovar 1/2a strain using the method described in chapter 4 the preparation contained 58 kDa and 35 kDa proteins. Although not formally identified the 58 kDa protein was assumed to be LLO. In immunoblot this protein was recognized by a convalescent serum raised against *L. monocytogenes* serovar 4b and the field sera showed similar

serological reactivity with both haemolysin preparations. There was no apparent recognition of the 35 kDa protein in immunoblot and the haemolysin preparation from serovar 1/2a was therefore used in an indirect ELISA to measure the titre of antibodies to LLO.

The generally higher optical density values obtained with the serovar 1/2a preparation precluded the definition of ELISA titres and a direct comparison of the results. The subtle differences in the optical density results are attributed to the different concentrations of antigen and it is maintained that the ELISA results were comparable using both haemolysin preparations. The anti-LLO antibodies do not appear to be serovar specific and the ELISA using LLO purified from *L. monocytogenes* serovar 4b is thus capable of detecting a serological response following infection by *L. monocytogenes* serovar 1/2a strains. It follows that the absence of anti-LLO antibodies from cases of listeric encephalitis cannot be attributed to the presence of serovar specific antibodies. Since the gene sequence of LLO is highly conserved between serotypes, with more than 99% homology in the amino acid sequences of LLO from representative strains of serovars 1/2a, 1/2c and 4b (Rasmussen *et al.*, 1991), there is likely to be serological cross reactivity between LLO from different *L. monocytogenes* serovars.

In field studies the sensitivity of the LLO ELISA for septicaemic infection was shown to be high and the specificity apparently 100%. However, the relationship between sensitivity and specificity do not permit a test to be absolute and an acceptance of a small number of false negatives is unavoidable. The assay has a sensitivity and specificity greater than those previously described (Seeliger, 1961; Hudak *et al.*, 1984; McLauchlin, 1988) and has a considerable advantage in detecting an antibody response to a major virulence factor produced during the process of intracellular multiplication. The use of LLO avoids the necessity of multiple serotype antigens and the ELISA is able to distinguish active infection from enteric carriage of non-haemolytic *Listeria* species. Application of the LLO ELISA to epidemiological studies with an examination for the development of anti-LLO antibodies in the sera of sheep kept under different farming conditions should at least establish the rate and extent to which animals are exposed to infection by *L. monocytogenes*. These studies may improve our understanding of the pathogenesis of various forms of listeric infection.

Since listeric encephalitis could not be diagnosed by serological means an alternative diagnostic method was sought and a brief examination of CSF samples for the presence of LLO was carried out. However, LLO was undetectable in CSF samples from five known cases of

listeric encephalitis or in a larger group of samples (results not shown). The failure to detect the antigen may be due to insensitivity of the assay or the result of LLO binding to cell membrane cholesterol and its rapid sequestration from the CSF. McLauchlin and Samuel (1989) described the use of an ELISA to detect soluble *Listeria* antigens in CSF samples from human cases of listeric meningitis but the sensitivity of the assay was only 27% and though this was improved by the use of a chemiluminescence method (Samuel *et al.*, 1990) this only identified five out of seven cases of listeriosis. It must be appreciated that in listeric encephalitis bacterial infection may not extend to the meninges and as these authors only examined two cases of encephalitis the sensitivity of the assays for this form of infection cannot be determined. In conclusion, although the LLO ELISA is useful for the diagnosis of listeric septicaemia and abortion the absence of a marked humoral antibody response in cases of encephalitis and the failure to detect LLO in CSF samples are not encouraging for the diagnosis of listeric encephalitis by immunological methods.

Chapter 8.

Serotyping and distribution of *Listeria* isolates from cases of ovine listeriosis.

Introduction

In the genus *Listeria* two pathogenic species exist: *Listeria monocytogenes* and *Listeria ivanovii*, and sixteen serovars can be identified on the basis of somatic and flagellar antigens (Rocourt and Seeliger, 1985; Seeliger and Jones, 1986). The majority of clinical infections are caused by *L. monocytogenes* serovars 1/2a, 1/2b and 4b (Seeliger, 1961; Gray and Killinger, 1966; Weis, 1975b; Kampelmacher and van Noorle Jansen, 1979; Ralovich *et al.*, 1986) but in veterinary publications serotyping of isolates has often been incomplete. A recent medical study described an association between serovar and the type of human clinical infection (McLauchlin, 1990) but other than the known relationship between *L. ivanovii* (serotype 5) and ovine abortion (MacLeod *et al.*, 1974; Rocourt and Seeliger, 1985) there has been no description of an association between *L. monocytogenes* serovar and category of animal listeriosis.

8.1 Identification and serotyping of *Listeria* strains

From January 1989 to May 1992, 117 *Listeria* isolates from a total of 85 cases of ovine listeriosis were received from the Scottish Agricultural College Veterinary Services Division. A case of listeriosis was defined either as an episode of infection where a *Listeria* sp. was isolated from a normally sterile site or where the organism was suspected by the clinician to be the cause of disease. When *L. monocytogenes* was isolated from the brain listeric encephalitis was confirmed by histopathological examination in 42 of 48 cases, the six exceptions being from one centre where the brains were not examined. Isolates were identified by the criteria described by Rocourt *et al.* (1983) using the methods described in section 2.4. *L. monocytogenes* strains were serotyped according to the Seeliger/Donker-Voet classification scheme (Seeliger and Jones, 1986) at the Division of Microbiological Reagents, Colindale.

Nine isolates, all recovered from cases of ovine abortion, were identified as *L. ivanovii* and the remaining 108 isolates as *L. monocytogenes sensu stricto*. Forty isolates taken from various organs of eight animals confirmed that isolates from individual cases of listeric septicaemia and encephalitis were consistently of identical serotype.

The following results are based upon serotyping of single isolates from 76 individual clinical cases involved in 50 separate incidents (an incident was defined as one or more cases of listeriosis confined to an individual flock). Overall, 49 (64.5%) of the *L. monocytogenes* isolates belonged to serovar 1/2a, 14 (18.4%) to serovar 4b and ten (13.2%) to serovar 1/2b. Serovar 3a was identified twice (2.6%) and serovar 4(not 4b) once (1.3%). Serovar 1/2a strains were isolated from all forms of clinical infection whereas serovar 1/2b strains were solely recovered from encephalitis cases. The overall distribution of the different serovars is shown in Table 8.1.

Table 8.1.

Distribution of *L. monocytogenes* serovars from 76 cases of ovine listeriosis

Category of infection	Number of cases	Serovar 1/2a	1/2b	3a	4b	4(not 4b)
Encephalitis	48	26	10	1	10	1
Septicaemia	8	6	0	0	2	0
Abortion	15	12	0	1	2	0
Iritis	5	5	0	0	0	0
Total	76	49	10	2	14	1

In only one of the 50 listeriosis incidents was there overlap of different clinical forms of disease when five iritis cases were also recorded with one abortion case. In ten incidents, involving 27 individuals, isolates from each case within the incident were of the same serovar. However, in one encephalitis incident two serovar 4b strains and a single 4(not 4b) serovar were isolated. In a second incident of encephalitis two strains belonged to serovar 1/2b, one to serovar 4b and one to serovar 1/2a and in a third encephalitis incident two isolates were identified as serovar 1/2a and three serovar 4b. The remaining 37 incidents all involved single cases and the distribution of serovars according to the clinical form of disease in the incidents is shown in Table 8.2.

Table 8.2.

Distribution of *L. monocytogenes* serovars from 50 incidents of ovine listeriosis

Category of infection	Number of incidents	Serovar 1/2a	1/2b	3a	4b	4(not 4b)
Encephalitis	32[a]	20	8	1	6	1
Septicaemia	4	3	0	0	1	0
Abortion	14	11	0	1	2	0
(Iritis[b]	1	1	0	0	0	0)
Total	50	34	8	2	9	1

[a] Of 32 encephalitis incidents three involved more than a single serovar. In one incident two serovar 4b strains and a single 4(not 4b) serovar were isolated. In the second incident two serovar 1/2b, one serovar 4b and one serovar 1/2a strains were isolated. In the third incident two isolates were identified as serovar 1/2a and three serovar 4b.

[b] One incident of abortion also involved five iritis cases.

8.2 Analysis of serovar distribution according to clinical condition

Fisher's exact test was used to examine the distribution of serovars according to the categories of disease in both cases and incidents. However, full analysis was not possible because of the limited numbers of isolates and the rarity of some serovars. No significant difference in the distribution of serovars 1/2a and 4b could be discerned but all serovar 1/2b strains were isolated from cases of encephalitis and statistical analysis suggested that serovar 1/2b strains were unevenly distributed between encephalitis and abortion cases in comparison to 1/2a strains (P=0.094).

However, as isolates were obtained from a number of individual cases within single incidents epidemiologically related strains may account for this heterogeneous distribution and the results were therefore analysed according to incident. This analysis supported the suggestion that 1/2a strains were more likely to be associated with abortion incidents than 1/2b strains (P=0.078) and in combined abortion and septicaemia incidents compared with encephalitis incidents alone the distribution was significantly different (P=0.037). These probabilities are not all statistically significant, at P<0.05, and since the data set is small

further serotyping of isolates is necessary to clarify whether these associations are real.

Discussion

The results of serotyping of *L. monocytogenes* strains are consistent with previous veterinary reports as serotype 1/2 was reported as the dominant isolate from animals in Hungary (Ralovich *et al.*, 1986) and in Great Britain between 1981 and 1984, 126 (67%) isolates from sheep belonged to serotype 1/2, 41 (22%) to serotype 4 and 19 (12%) were *L. ivanovii* (Audurier *et al.*, 1986). In Norway an equal distribution of serogroups 1 and 4 were isolated from ovine and caprine encephalitis cases but only serogroup 1 strains were isolated from caprine abortion material (Kummeneje, 1975). The identification of *L. ivanovii* from nine cases of abortion is in agreement with the reports of Rocourt and Seeliger (1985) and Audurier *et al.* (1986) that the organism occurs infrequently and is almost solely associated with ovine abortion. The receipt of 63% of isolates from encephalitis cases is consistent with reports that this is the major clinical manifestation of ovine listeriosis in UK (Anon., 1983) and as previously described (Wilesmith and Gitter, 1986) overlap of different forms of listeriosis within the same flock is a relatively rare occurrence.

Serovar 1/2a strains predominated in this study which may be linked to its occurrence in silage, a foodstuff that is commonly associated with listeric infections of sheep (Wilesmith and Gitter, 1986). The result is in contrast to the results of a medical study in which serovar 4b was most commonly isolated from human disease in Scotland (Campbell, 1990). However it is now known that many of these cases were epidemiologically related (McLauchlin *et al.*, 1991) and thus previously unrecognized factors possibly account for the predominance of serovar 4b in human disease.

The results suggest that there is no difference in the distribution of *L. monocytogenes* serovars 1/2a and 4b but that 1/2b has an association with ovine encephalitis. Since experimental studies have shown the pathogenesis of septicaemia and abortion to be similar (Smith *et al.*, 1970; Njoku and Dennis, 1973) and distinct from encephalitis (Asahi *et al.*, 1957; Urbaneck, 1962b; Barlow and McGorum, 1985) the distribution of serovars in encephalitis incidents was compared with the distribution in combined abortion and septicaemia incidents. This confirmed a significant difference in the distribution of serovar 1/2a and 1/2b strains. McLauchlin (1990) suggested that serovar 1/2b was less pathogenic than serovars 1/2a or 4b and the non-random distribution of the serovar may be associated with an ability to cause

disease in an immunologically privileged site such as the brain but an inability to cause abortion or septicaemia through systemic infection. The rarity with which serovars 3a and 4(not 4b) occurred and the absence of serovar 1/2c are consistent with the assertion that these serovars are less pathogenic than serovars 1/2a, 1/2b and 4b (McLauchlin, 1990).

Recent studies by multilocus enzyme electrophoresis (MEE) have demonstrated two major clonal divisions of *L. monocytogenes* associated with flagellar serotype (Piffaretti *et al.*, 1989; Bibb *et al.*, 1990) and there is also evidence that these divisions possess different gene sequences for the recognised virulence factor listeriolysin O (Rasmussen *et al.*, 1991). Examination for a difference in the distribution of flagellar a and b strains was biased by the non-random distribution of serovar 1/2b strains but no clear association of flagellar a serovar strains with abortion incidents or combined abortion and septicaemia incidents was found when compared to the distribution of flagellar b serovars (P=0.10 and P=0.12 respectively, Fisher's exact two tailed test).

Overall the findings may be taken to indicate differences in the distribution and prevalence of serovars which are possibly associated with the virulence potential of individual strains of *L. monocytogenes* or epidemiological factors such as their frequency in feedstuffs. The results are consistent with pyrolysis mass spectrometry (PyMS) which has shown closely related or identical strains of *L. monocytogenes* serovar 1/2a to be associated with different outbreaks of ovine listeriosis (Low *et al.*, 1992) and the demonstration that closely related serovar 4b strains have caused geographically diverse epidemic disease in man (Piffaretti *et al.*, 1989; Bibb *et al.*, 1990). The findings may in part account for the rarity with which different listeric conditions occur in single flocks (Ladds *et al.*, 1974; Sullivan, 1985; Wilesmith and Gitter, 1986).

General Discussion

Chapter 9.

General discussion and future developments.

The principal objectives of the study were to identify and isolate the somatic antigen determinants of *Listeria monocytogenes* and to develop diagnostic assays for listeriosis. In attempting to identify the somatic antigen determinants monoclonal antibodies (mAbs) were successfully raised against heat-killed *L. monocytogenes* but these were neither specific for the genus nor did they detect all strains within a serotype. Other reports have described the development of mAbs against somatic antigens but none of these studies, using immunization with crude antigen preparations, has helped in elucidating the nature of the somatic antigen determinants of the *Listeria* genus.

The mAbs described by McLauchlin *et al.* (1986b) are serotype specific and recognize an undefined carbohydrate antigen in the cell wall (McLauchlin, 1988). Ziegler and Orlin (1984) produced a panel of mAbs, the majority of which recognized antigens on heat-killed *L. monocytogenes* cells, but neither the antigenic epitopes nor the cross reactivity of the mAbs with other organisms were defined. Siragusa and Johnson (1990) used three immunization

protocols, including the use of heat-killed *L. monocytogenes*, and although 27 mAbs were produced and characterized only one was shown to be genus specific. The antigen detected was a cell bound protein of 18.5 kDa molecular mass. This group also produced another anti-*Listeria* mAb which reacted with a number of proteins in SDS-PAGE (Bhunia *et al.*, 1991) and most recently a mAb which recognizes a protein of 66 kDa molecular mass and is specific for *L. monocytogenes* (Bhunia and Johnson, 1992). Monoclonal antibodies against *Listeria* flagellar antigens have been produced (Farber and Spiers, 1987a; Peel, 1987; Butman *et al.*, 1988) and used in the development of isolation techniques for the organism (Farber and Spiers, 1987a; Mattingly *et al.*, 1988; Skjerve, 1990). However no serological assay based upon any of these mAbs has been described.

The mAbs produced in this thesis were of academic interest and although unsuccessful for use in a serological assay they were partially characterized. The recognized antigen had many of the characteristics of lipoteichoic acid (LTA) but further analyses such as thin layer chromatography, analysis and quantification of sugars by gas chromatography or the use of nuclear magnetic resonance would be necessary for confirmation. Assuming the antigen is LTA, deacylation would permit the determination of whether the mAbs recognise carbohydrate

substituents of the side chain or epitopes within the acyl lipid anchor. The failure to detect the antigenic epitope in all *Listeria* strains is inconsistent with the results of biochemical studies which have suggested identical LTA structures in diverse *Listeria* serotypes (Hether and Jackson, 1983; Uchikawa *et al.*, 1986b; Ruhland and Fiedler, 1987). Since the synthesis of LTA is not in general affected by growth conditions (Knox and Wicken, 1973), and if the mAbs do recognize LTA, the results must be attributed either to inaccessibility of the antigenic epitope at the cell surface or to unrecognized differences in biochemical structure of LTA. These differences may be due to variations in carbohydrate substituents or the steric conformation of the epitope.

Although LTA is a component of the bacterial cytoplasmic membrane it is a surface antigen in Group F *Lactobacillus fermenti* (Knox *et al.*, 1970), Group D *Streptococci* (Knox and Wicken, 1973) and some *Bacillus* species (Burger, 1966). Surface reactivity of these membrane components is generally ascribed to a porosity of the bacterial cell walls which allows antibody antigen reactions to occur (Burger, 1966; Knox and Wicken, 1973). The assertion that the cross reacting "Rantz" antigen common to a number of Gram positive bacteria including *L. monocytogenes* (Rantz *et al.*, 1956; Neter *et al.*, 1960) is

LTA is not incompatible with the proposition that the mAbs recognise LTA yet only recognize *Listeria* and *Bacillus* species. Since cross reactions are attributable to antibodies to the common glycerophosphate backbone (Knox and Wicken, 1973) it is assumed that the mAb binding epitope is distinct from this part of the molecule. The role of LTA as the common *Listeria* somatic antigen III can only be clarified by the production of polyclonal sera to purified LTA and an examination of their reactivity with phenol extracts from representatives of all *Listeria* serovars. These investigations were not considered to be useful for the development of a specific serodiagnostic assay.

It seems remarkable that immunization with a crude antigen preparation resulted in a panel of mAbs from which five chosen recognized the same antigen and at least three of these competed for binding to the same epitope. Though this response may be a feature peculiar to the murine immune system there are reports of T-cell independent, B-cell mitogens in *Listeria* cell walls (Cohen *et al.*, 1975; Campbell *et al.*, 1976; Paquet *et al.*, 1986) and it is possible the mAbs were produced in response to this mitogen.

Attempts were made to utilize the mAbs in a serodiagnostic assay but the experimental results were inconsistent and the reagents were judged to be unsuitable for a reliable assay. On the other hand listeriolysin O (LLO), purified to apparent homogeneity by cation exchange chromatography, was recognized as having unique properties for serodiagnosis. Although previous studies had shown antigenic relationships with other sulphydryl-activated toxins, no recognition of streptolysin O (SLO) or clostridial toxin preparations occurred with a polyclonal hyperimmune rabbit serum raised against purified LLO. Similarly the field sera which recognized LLO in immunoblots and ELISA did not recognize SLO in immunoblots. Berche *et al.* (1990) showed that anti-LLO antibodies remain following the absorption of sera with SLO and Mengaud *et al.* (1987) described differences in the gene sequences of LLO, SLO and pneumolysin. Thus it seems that there are specific antigenic epitopes within LLO which are dominant epitopes and can be recognized in serological assays.

In the assessment of all the serological assays difficulties were encountered as the clinical responses of the animals' following oral dosing and subcutaneous injection with *L. monocytogenes* were subtle. As a bacteraemia could not be detected after oral dosing there was no "gold standard" result by which to judge the

sensitivity of the assays. Analysis of the serological results from the experimental studies indicated that the detection of anti-LLO antibodies by immunoblotting was a consistent indicator of both oral and subcutaneous challenges. However, it must be recognized that in using this technique to assess whether animals had been "infected" could lead to bias in assessing the other assays. Miettinen and Husu (1991) independantly reported that goats seroconverted to LLO after experimental challenge with *L. monocytogenes* and the objective of developing a serological assay using purified LLO was therefore pursued. An ELISA was developed which performed well in comparison with immunoblotting, was simple to use and had a sensitivity of 82% for the putative systemic infections. Though the presence of anti-LLO antibody cannot be taken as a reflection of immunity the LLO ELISA is apparently a reliable indicator of infection and sufficiently sensitive to detect antibody responses arising after oral challenge and systemic infection.

The setting of the positive/negative discrimination level is a crucial element in the design of all antibody assays (de Savigny and Voller, 1980). Under the standard LLO ELISA conditions the thirty six experimental sera taken prior to challenge produced a mean absorbance value of 0.012 ± 0.011 (SD). Though a positive/negative "cut-off"

is often set at the mean absorbance plus three times the standard deviation the derived value of 0.045 was felt to be, in practical terms, too low. Antibody concentrations in the population are distributed with a positive skew (de Savigny and Voller, 1980) and as the computer programme was designed to operate with a positive/negative "cut-off" of 0.1 it was decided to accept the value of 0.1 as an arbitary division between positive and negative. It was recognised that this level may produce some false negative results but until further samples are tested and confidence limits set a more accurate division cannot be established.

In field studies the detection of antibodies to LLO was shown to be useful for diagnosis of both septicaemic and abortion forms of listeriosis but in cases of listeric encephalitis anti-LLO titres were negative or inconsistent with a recent septicaemic episode. Loken and Gronstol (1982) and Peel (1987) using crude *Listeria* antigens also failed to detect any humoral antibody reponses in cases of caprine and ovine listeric encephalitis. Thus the accumulated evidence indicates that there is no serological response to listeric antigens in encephalitis cases and it is therefore impracticable to diagnose the condition through the measurement of serum antibodies.

The absence of a serological response in encephalitis cases may be due to infection being so recent that there has been no antibody production or that infection is of such a low grade nature and confined to the CNS that there is little or no antigenic stimulation of B-lymphocytes. Alternatively the animals may be so immunosuppressed they cannot mount an antibody response. Field and experimental reports have indicated a long incubation period for listeric encephalitis (Asahi *et al.*, 1957; Borman *et al.*, 1960; Urbaneck, 1962b; Barlow and McGorum, 1985; Low and Renton, 1985) and the first suggestion is unlikely. Though immunosuppression has been suggested as predisposing to listeric encephalitis (Gronstol, 1980b; 1980c; Gronstol and Overas, 1980a; 1980b) no decrease in serum IgG concentration was found in silage fed sheep (Gitter *et al.*, 1986a) and pregnant ewes are known to seroconvert to clostridial and pasteurella vaccines. It is thus improbable that immunosuppression has depressed the humoral antibody responses to *L. monocytogenes*. The low anti-LLO titres in listeric encephalitis cases are therefore assumed to be a consequence of the confinement of the organism to the CNS with little antigen presentation to B-cell lymphocytes. This is supported by the failure to detect LLO in CSF samples from confirmed listeric encephalitis cases and though the finding does not clarify the route of infection the implication is that listeric

encephalitis does not develop in those ewes sensitized by a previous immune response as suggested by Barley (1990).

Alternative diagnostic methods for listeric encephalitis might include the demonstration of the organism or bacterial products in CSF samples. McLauchlin and Samuel (1989) described the use of an ELISA to detect soluble *Listeria* antigen in CSF samples from human cases of listeric meningitis. However, the sensitivity of the assay was poor and though this was improved by the use of a chemiluminescence assay (Samuel *et al.*, 1990) both assays are unreliable. Oligonucleotide primers based on the *L. monocytogenes* invasion associated protein (*iap*) gene (Kuhn and Goebel, 1989; Kohler *et al.*, 1990) with amplification by polymerase chain reaction (PCR) have been used to detect very low numbers of bacteria in CSF samples. However, with clinical specimens the sensitivity of the assay was only 71% and four false positives occurred due to contamination of the samples (Jaton *et al.*, 1992). The unreliable results and the inherently high cost of molecular techniques make it unlikely that these methods will be applied in the veterinary field. Thus although examination of the cellular components of the CSF may be indicative of listeric encephalitis (Rebhun and deLahunta, 1982; Scott, 1992) there is still no accurate antemortem diagnostic method.

In the experimental studies oral dosing and injection of lambs with massive numbers of virulent *L. monocytogenes* resulted in no clinical signs of enteritis or septicaemia. Although circumstantial evidence was presented that oral dosing resulted in invasion and the development of immunity, the site of invasion and extent of infection remains unknown. In laboratory animals it is difficult to reproduce a lethal infection through oral dosing with massive numbers of *L. monocytogenes* (Racz *et al.*, 1972; Zachar and Savage, 1979; MacDonald and Carter, 1980; Roll and Czuprynski, 1990; Marco *et al.*, 1992) and similarly after the subcutaneous injections some lambs were not detectably bacteraemic. Thus there is evidence to support the comment of Harty and Bevan (1992) that innate immune mechanisms can cope with large doses of virulent *L. monocytogenes*. In the sheep studies, though the infectious dose for other strains of *L. monocytogenes* was not determined, the absence of a detectable bacteraemia and lack of clinical signs after oral dosing with 10^{10} c.f.u. viable *L. monocytogenes* suggests that factors other than dose are important in the development of enteritis and septicaemia.

Recent field investigations have identified an enteritis with microabscessation in the bowel associated with *L. monocytogenes* infection but the pathogenesis of this form of infection is unclear. In contrast to the experimental

results, field studies have shown that faecal excretion of *L. monocytogenes* is common after introduction to silage feeding (Gronstol, 1979a; Loken *et al.*, 1982). Since a period of starvation and neutralization of gastric pH were necessary to produce listeric enteritis in guinea-pigs (Racz *et al.*, 1972) it is important that the iron content and abomasal buffering capacity of silage are examined for roles in the development of enteric carriage and listeric disease. Currently the oral challenge system is in use to examine the pathogenesis of the septicaemic form of infection and to study the cell-mediated immune reponses to LLO in infected animals. These studies have confirmed that lambs develop a cell-mediated immune response to *Listeria* after oral dosing with *L. monocytogenes* and that infected animals have circulating lymphocytes which recognize LLO in lymphocyte transformation tests.

An understanding of the pathogenesis of listeric encephalitis was not a major objective of this study but some conclusions can be drawn from the occurrence of clinical cases amongst the experimental animals. As neurological symptoms occurred in six animals after seroconversion to listeric antigens had been demonstrated the studies suggest that the protective mechanism against bacteraemia, acquired after exposure to viable *L. monocytogenes,* does not confer protection against

neurological disease. Further the variation in incubation period from 48 hours to 61 days is indicative of the possibility of latency of the organism and a failure of the host to effectively eliminate the bacterium from the brain. In listeric encephalitis the pathway for infection of the brain may be the cranial nerves (Asahi *et al.*, 1957; Urbaneck, 1962a; Barlow and McGorum, 1985) and in this study there is circumstantial evidence based upon the topography of the lesions for infection of the spinal cord arising via the spinal nerves after subcutaneous injection on the side of the neck. Although the failure to develop a diagnostic assay for encephalitis is a disappointment further studies into the development of specific anti-LLO antibodies in individuals under different farming conditions may enlighten our understanding of the pathogenesis and epidemiology of listeric encephalitis.

It is crucial that the role of cell-mediated immunity in the study of the pathogenesis, diagnosis and epidemiology of ovine listeriosis is no longer ignored. The recent development of a gamma interferon ELISA (Rothel *et al.*, 1990) offers an attractive alternative to the labour intensive lymphocyte transformation tests and it is now feasible to use LLO, purified by cation exchange chromatography, as an antigen in these assays. It has recently been shown that in mice cytotoxic T-cells are

highly effective in protecting against listeric disease (Bouwer *et al.*, 1992; Harty and Bevan, 1992) and a fascinating possibility is that those sheep suffering clinical encephalitis have an aberrant T-cell response which is incapable of overcoming infection. It is now feasible to use cell markers to define the pathological lesions in field cases of listeric encephalitis and together with the measurement of the animals' cell-mediated immune responses and the *L. monocytogenes* specific mAb of Bhunia and Johnson (1992) there is an opportunity to localise the organism *in situ* and to define the exact nature of the host's immune response. Until these studies have been performed it is inhumane to suggest the destruction of more animals in the search for a reliable model of encephalitic disease.

Evidence for a non-random distribution of serovars of *L. monocytogenes* from different forms of clinical disease was produced and in future veterinary studies it is essential that full serotyping of *L. monocytogenes* isolates is carried out to determine if particularly virulent strains of *L. monocytogenes* exist or if the distribution of clinical isolates simply represents those strains to which animals are most commonly exposed. It is thus important that *L. monocytogenes* isolates from the agricultural environment and silage are collected and the distribution of the characterized strains is defined.

Characterization of *L. monocytogenes* isolates by multilocus enzyme electrophoresis (MEE) has produced evidence for two major clonal divisions (Piffaretti *et al.*, 1989; Bibb *et al.*, 1990) coincidental with the presence or absence of the flagellar C antigen. Since there is a possible association between serological type and virulence (McLauchlin, 1990) it is clear that a comparison of strains of the same serovar and MEE electrophoretic type should be conducted to define their virulence in suitable animal models.

Bacterial strains can be screened for pathogenicity in immunocompromised mice (Stelma *et al.*, 1987; Conner *et al.*, 1989; Tabouret *et al.*, 1991) and using this technique isolates from a variety of sources have been examined and avirulent, haemolytic strains of *L. monocytogenes* identified. Though Conner *et al.* (1989) report avirulent, haemolytic strains from clinical specimens Tabouret *et al.* (1991) only identified avirulent, haemolytic strains from environmental specimens and these belonged to serovar 1/2a and 3a. It should be noted that this immunocompromised mouse model is not very discriminatory as 93% and 88% of isolates were respectively classified as virulent and the method relies on intraperitoneal injection which ignores other relevant aspects of virulence such as adhesion to intestinal cells and invasion of the host.

Audurier *et al.* (1980) stated that it is impracticable to assess the virulence of strains through dosing by a single route. Therefore in future studies it is suggested that an *in vitro* method (Pine *et al.*, 1991) for the measurement of virulence, which compares well with the immunocompromised mouse model, is used to screen all strains which have been characterized by serotyping and MEE. Thereafter it is envisaged that the pathogenic capability of these strains will only be distinguished by both oral dosing and intravenous challenge of mice with liver and spleen counts being performed at three and six days. Since the genetic regulation of *Listeria* pathogenicity has been clarified and virulence mechanisms and molecular determinants of pathogenicity have been reviewed (Portnoy *et al.*, 1992) a consideration of these reports shows that it is possible to examine these strains of *L. monocytogenes* for the possession of virulence associated genes and virulence characteristics.

Listeriolysin O is an essential virulence factor (Cossart *et al.*, 1989) but the level of LLO production *in vitro* is not proportional to virulence (Kathariou *et al.*, 1988) and since LLO production is affected by mutations outside the haemolysin gene (*hylA*) (Leimeister-Wachter et al., 1989) other factors must be of importance in determining virulence. A number of variant strains of *L. monocytogenes* exist which lack both haemolytic activity

and other putative virulence determinants such as: lecithinase production and the ability to enter mammalian cells (Kathariou *et al.*, 1990). A factor which positively regulates expression of LLO in pathogenic strains has been identified and plasmids harbouring the regulatory gene, *prfA*, increased production of both LLO and other proteins, and transformed some nonhaemolytic mutant strains into haemolysin producers (Leimeister-Wachter *et al.*, 1990). The *prfA* gene encodes a protein of 27 kDa molecular mass which has no significant homology to other bacterial regulatory proteins (Leimeister-Wachter *et al.*, 1990). Further studies have shown the *prfA* gene to be specific to *L. monocytogenes*, to regulate its own synthesis and to be a positive regulator of multiple virulence determinants involved in invasion, intracellular bacterial cell motility and cell to cell spread (Mengaud *et al.*, 1991a; Chakraborty *et al.*, 1992).

Electron microscope studies of infected tissue culture cell lines have shown that cytoplasmic *L. monocytogenes* become encapsulated by actin filaments that rearrange to form a tail which propels the bacterium to the cell surface and into neighbouring cells (Tilney and Portnoy, 1989; Mounier *et al.*, 1990). The speed of movement is rapid and may approach 1.5 μm sec^{-1}. Since chloramphenicol blocks actin assembly this movement is at

least partly under bacterial control (Dabiri *et al.*, 1990). However, actin polymerization is not the sole factor involved in intracellular movement as an avirulent transposon mutant also polymerizes actin but does not move intracellularly (Kuhn *et al.*, 1990).

Tilney *et al.* (1990) produced a mutant strain which was unable to polymerize actin and Kocks *et al.* (1992) used transposon mutagenesis to demonstrate the second gene, termed *actA*, of the operon down stream of the *hylA* gene encodes a surface protein with a calculated molecular mass of 67 kDa and an apparent molecular mass of 90 kDa in SDS-PAGE. The function of the protein is obscure but it is clearly involved in actin polymerization and its structure has strong regional homology to that of the cytoskeletal protein vinculin (Domann *et al.*, 1992). Strains with mutations within the *actA* gene do not polymerize actin, express no lecithinase activity and have markedly reduced virulence (Kocks *et al.*, 1992; Domann *et al.*, 1992).

Entry of *L. monocytogenes* to mammalian cells is associated with production of a 80 kDa molecular mass protein "internalin" (Gaillard *et al.*, 1991). Transposon mutagenesis of the internalin gene (*inlA*) led to a failure of the organism to invade epithelial cells and introduction of the gene to *L. innocua* enabled this

noninvasive bacterium to enter cells. Sequencing of the gene predicts a protein with a cytoplasmic membrane anchor at the C-terminus and a structure similar to surface proteins of Gram positive cocci with internal repeat sequences reminiscent of *Streptococcus pyogenes* M protein. The gene is part of a gene family and under nonstringent conditions a gene probe detected several related genes in *L. monocytogenes*, *L. ivanovii* and *L. innocua*. The possibility of considerable functional and antigenic variability in the *inlA* gene may be of importance in the comparison of strains. The protein termed P60 (Kuhn and Goebel, 1989) may also have a role in cell invasion but its function remains unclear. Although rough forms of *L. monocytogenes* lacking P60 are unable to enter cells the protein is unrelated to internalin (Gaillard *et al.*, 1991) and P60 is expressed by the noninvasive transposon mutants of Kathariou *et al.* (1990).

Adjacent to the *hylA* gene the phosphatidylinositol-specific phospholipase C (PI-PLC) gene encodes a protein of approximately 36 kDa molecular mass which has partial homology to the PI-PLC of *Bacillus thuringiensis* and *B. cereus* (Camilli *et al.*, 1991; Leimeister-Wachter *et al.*, 1991; Mengaud *et al.*, 1991b). PI-PLC activity is only present in pathogenic strains and it is suggested mutant strains are defective for cell to cell spread due to PI-

PLC acting in concert with LLO for membrane lysis (Camilli *et al.*, 1991). Transcribed with LLO by divergent nonoverlapping promoters the gene is the first gene of an operon upstream of *hylA* which is only present in the pathogenic species *L. monocytogenes* and *L. ivanovii* (Gormley *et al.*, 1989). An assay for virulence based upon the detection of PI-PLC activity has been described (Notermans *et al.*, 1991) but mutations within the PI-PLC gene exert polar effects upon *prfA*, the second gene of the operon, and it is therefore difficult to assess the role of PI-PLC in virulence (Camilli *et al.*, 1991; Leimeister-Wachter *et al.*, 1991; Mengaud *et al.*, 1991b).

Historically a lecithinase activity has been ascribed to *L. monocytogenes* (Fuzi and Pillis, 1962; Jenkins and Watson, 1971) and recently a zinc dependant phospholipase of 29 kDa molecular mass which possesses lecithinase activity has been purified from the organism (Geoffroy *et al.*, 1991). The protein is distinct from the PI-PLC and all pathogenic strains of *L. monocytogenes* examined expressed lecithinase activity. The lecithinase has weak haemolytic activity against erythrocytes of some mammalian species but no activity against those of sheep. Immunoblotting of *L. monocytogenes* culture supernatant fluids has identified, in addition to the 29 kDa molecular mass lecithinase, an antigenically related

doublet of approximately 33 kDa molecular mass. The gene encoding the lecithinase has been identified as the third of the operon downstream of *hylA*, the encoded protein is related to the phospholipase C of *B. cereus* and the alpha toxin of *Clostridium perfringens* and is apparently involved in lysis of the double membrane which occurs during cell to cell spread (Vazquez-Boland *et al.*, 1992). The actin nucleating protein is encoded by the second gene (*actA*) of this operon and the first gene has been defined as encoding a metalloprotease with a predicted molecular mass of 57.4 kDa and a mature size of 34.5 kDa (Mengaud *et al*, 1991c). The metalloprotease is involved in maturation of the 33 kDa molecular mass lecithinase precursor to the mature 29 kDa protein (Raveneau *et al.*, 1992). Transposon mutants within the gene are strongly impaired for virulence and this region of the lecithinase operon is specific for *L. monocytogenes* (Gormley *et al.*, 1989; Vazquez-Boland *et al.*, 1992).

Sequencing of the haemolysin and metalloprotease genes (Mengaud *et al.*, 1991c; Rasmussen *et al.*, 1991) and restriction fragment length polymorphisms within the *hylA*, metalloprotease, *prfA* and *iap* genes (Vines *et al.*, 1992) have shown differences which are consistent with the two major clonal divisions defined by MEE. It is envisaged that strains which have been characterized on the basis of serotype, MEE electrophoretic type and

virulence can be examined phenotypically for the production of: LLO, lecithinase, and PI-PLC together with the ability to spread from cell to cell. Using PCR techniques with defined DNA primers and restriction enzyme digestion (Vines *et al.*, 1992) will allow an assessment of whether restriction fragment length polymorphisms exist in the virulence associated genes of these strains. However it must be recognized that, as single amino acid substitutions within LLO (Michel *et al.*, 1990) and subtle amino acid differences within the thiol activated toxins of *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* (Vazquez-Boland *et al.*, 1989b; Haas *et al.*, 1992) have profound effects upon toxic activity, differences between strains may be slight and require detailed examination for their definition.

In summary, I would suggest two major areas of study are pursued. Firstly, the nature of the immune response and the cell types involved in the pathological lesions of field cases of listeric encephalitis should be defined. Secondly, *L. monocytogenes* isolates from environmental sources and clinical specimens should be collected and characterized by serotyping and MEE. The distribution of strains can be defined and subsequently they can be examined for the possession of known virulence characteristics and their virulence can be compared in suitable animal models.

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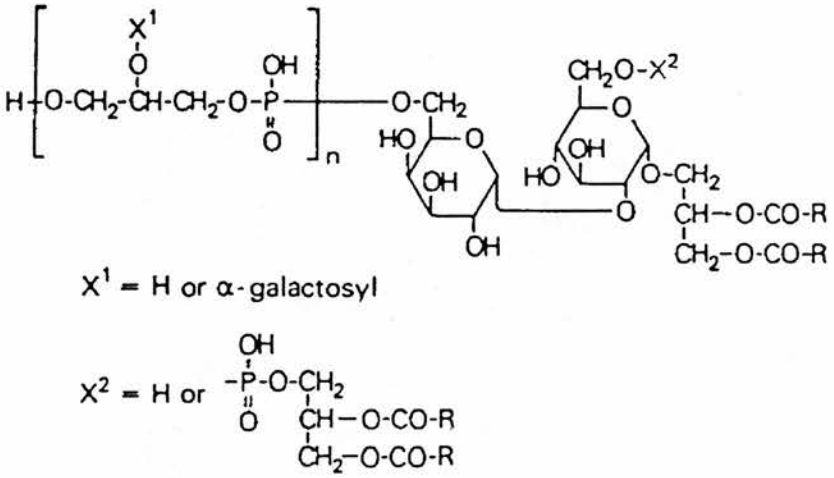
Appendices

Appendix 1.

Structure of *Listeria* lipoteichoic acid.

(according to Uchikawa *et al.*, 1986b)



X^1 = H or α-galactosyl

X^2 = H or

R groups represent the hydrocarbon portion of long-chain fatty acids.

Appendix 2.

Comparison of ELISA optical densities obtained with field sera using as antigens LLO purified from *L. monocytogenes* serovars 1/2a or 4b.

Flock 2.
(Table 7.2).

Animal	serovar 1/2a LLO	serovar 4b LLO
A1.	0.22	0.52
A2.	0.30	0.29
A3.	0.64	0.47
A4.	0.09	0.09
A5.	1.01	1.35
A6.	0.72	0.51
B1.	0.03	0.03
B2.	0.13	0.12
B3.	0.70	0.89
B4.	1.16	1.07
B5.	0.35	0.34
B6.	1.13	1.04
C1.	0.03	0.04
C2.	0.04	0.05
C3.	0.19	0.31
C4.	0.69	0.76
C5.	0.16	0.10
C6.	1.44	1.70
D1.	0.04	0.04
D2.	0.00	0.05
D3.	0.07	0.06
D4.	0.20	0.12
D5.	0.08	0.07
D6.	0.03	0.05

Confirmed listeric encephalitis cases
(Table 7.6).

Animal	serovar 1/2a LLO	serovar 4b LLO
1.	0.12	0.09
2.	0.28	0.33
3.	0.24	0.13
4.	0.30	0.18
5.	0.13	0.05
6.	0.20	0.23
7.	0.21	0.11
8.	0.04	0.02
9.	0.53	0.32
10.	0.00	0.00
11.	0.03	0.01
12.	0.10	0.11
13.	0.13	0.09
14.	0.25	0.09
15.	0.16	0.10
16.	0.04	0.00

Listeric abortion case
(Table 7.7).

Animal	serovar 1/2a LLO	serovar 4b LLO
Acute sample	0.73	0.73
Convalescent sample	0.27	0.33

Clinically normal sheep and those suffering diseases other than listeriosis
(Table 7.8).

Animal	serovar 1/2a LLO	serovar 4b LLO
1.	0.05	0.05
2.	0.07	0.13
3.	0.05	0.02
4.	0.01	0.00
5.	0.00	0.01
6.	0.12	0.02
7.	0.06	0.04
8.	0.08	0.05
9.	0.05	0.04
10.	0.08	0.04
11.	0.06	0.04
12.	0.02	0.02

Appendix 3.

Publications arising from the thesis.

Low.J.C., and Donachie.W. (1991). Clinical and serum antibody responses of lambs to infection by *Listeria monocytogenes*. Research in Veterinary Science. 51. p.185-192.

Low.J.C., Chalmers.R.M., Donachie.W., Freeman.R., McLauchlin.J., and Sisson.P.R. (1992). Pyrolysis mass spectrometry of *Listeria monocytogenes* isolates from sheep. Research in Veterinary Science. 53. p.64-67.

Low.J.C., Davies.R.C., and Donachie.W. (1992). Purification of Listeriolysin O and development of an immunoassay for diagnosis of listeric infections in sheep. Journal of Clinical Microbiology. 30. p.2705-2708.

Low.J.C., Wright.F., McLauchlin.J., and Donachie.W. Serotyping and distribution of *Listeria* isolates from cases of ovine listeriosis. (Submitted for publication to The Veterinary Record, January 1993).

esearch in Veterinary Science 1991, 51, 185–192

Clinical and serum antibody responses of lambs to infection by *Listeria monocytogenes*

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Oral dosing of lambs with 1×10^{10} colony forming units of *Listeria monocytogenes* daily for three days produced no clinical signs but protected the animals against bacteraemia following subsequent homologous subcutaneous challenge. Following the subcutaneous injections, comparison with controls revealed significantly lower rectal temperatures and a significant difference in positive blood cultures. In both groups signs of systemic illness were unremarkable. However, two and 10 days after the subcutaneous challenges neurological signs developed in two lambs. *L monocytogenes* was isolated from the brain of one lamb and histopathological lesions of listeric encephalomyelitis were demonstrated in both. After oral infection antibodies to *L monocytogenes* whole cell antigen were detectable in serum agglutination tests and by ELISA. Serological responses to flagellin were examined by ELISA and to listeriolysin O by immunoblotting. The responses of the animals to flagellin were weak and inconsistent, but antibodies to listeriolysin O were detectable after both oral and subcutaneous challenge. The subclass of antibody involved in this response was shown to be predominantly IgG_1.

LISTERIC infections occur worldwide and in a variety of animals including man (Gray and Killinger 1966). Recently there appears to have been an increasing prevalence in both sheep (Anon 1989) and man (McLauchlin 1987) and the disease has attained considerable significance in both species. Though new advances in isolation techniques, together with improvements in taxonomy and typing (McLauchlin 1987) have extended our knowledge of listeria, both immunological and epidemiological aspects of infection remain poorly understood.

Diagnosis and epidemiological investigations would be considerably improved by the development of a simple and reliable serological test. Many of the tests currently available are non-specific because crude bacterial suspensions are used as antigens. In the widely used serum agglutination test cross-reacting antigens from *Staphylococcus, Enterococcus* and other Gram-positive species give rise to listeria agglutinating antibodies in healthy animals (Osebold and Sawyer 1955, Seeliger 1961, Gray and Killinger 1966). Furthermore, a large number of serotypes is contained within the species *L monocytogenes* and antigens from each must be used when performing serological examinations (Seeliger 1961).

Two proteins, flagellin and listeriolysin O, have now been characterised and may offer alternatives to crude bacterial suspensions. Flagellar antigens are part of the serotyping system for *Listeria* as described by Seeliger and Hohne (1979). The purified flagellin is a protein with a molecular weight in the region of 29 kDa (Peel et al 1988). Listeriolysin O is a recognised virulence factor for *L monocytogenes* (Gaillard et al 1986), and is produced by all strains (Geoffroy et al 1989). It is a thiol-activated toxin with a molecular weight of 58 kDa and structurally related to haemolysins produced by other Gram-positive organisms (Geoffroy et al 1987).

There is evidence that foodstuffs are commonly contaminated with listeria and that oral infection is an important route of infection in man and sheep (Gray 1960, Schlech et al 1983, Low and Renton 1985, James et al 1985). In this study the authors examined the clinical responses of sheep following primary oral and secondary subcutaneous challenge with virulent *L monocytogenes* and the subsequent humoral antibody responses to crude and defined listeric antigens.

Materials and methods

Bacteria

Listeria monocytogenes, Special *Listeria* Culture Collection strain 2375 serovar 4b (National Collection of Type Cultures strain 10527) was used throughout. The organism was maintained in a lyophilised state and also on Dorset egg agar slopes at 4°C. Before challenge the organism was passaged through mice. Passage was effected by intraperitoneal inoculation of a Swiss white mouse with approximately 1×10^9 colony forming units of *L monocytogenes* from an 18

hour culture. *L monocytogenes* was recovered from the liver of the mouse 24 hours after infection.

Challenge inocula were prepared by growing bacteria in 5 litres of brain heart infusion broth (Oxoid) for 18 hours at 37°C. The organism was pelleted by centrifugation at 2400 *g* for one hour at 4°C and washed three times in phosphate buffered saline, pH 7·4 (PBS). The final pellet was resuspended in a final volume of 25 ml PBS and 1 ml samples were maintained at −70°C until use. Throughout, the purity of the organism was monitored and identity confirmed by its cultural characteristics, Gram staining, morphology, tumbling motility at room temperature, catalase production and sugar fermentation reactions. Viable counts of two samples were made before freezing and subsequently from one sample at the time of each challenge.

Antigens

Flagellin for use in enzyme linked immunosorbent assays (ELISA) was prepared by the method of Peel (1987). Listeriolysin O for immunoblotting was precipitated with cholesterol by the method of Vazquez-Boland et al (1989). Crude somatic antigen was prepared from bacteria grown in tryptic soy broth (Gibco) for 18 hours at 37°C. The organisms were killed by heating to 100°C for one hour and the cell pellet collected by centrifugation of the bacterial suspension at 2400 *g* for one hour at 4°C. The bacterial cells were washed and resuspended three times in PBS and the final pellet lyophilised.

Serum agglutination tests

L monocytogenes serovar 4b O cell suspensions (Behringwerke) were used as described by the manufacturers.

Flagellin ELISA

Listeria flagellin at a concentration of 1 μg protein ml^{-1} in 0·05 M carbonate/bicarbonate buffer was added in 100 μl volumes to the wells of Dynatech M129A microtitre plates. Covered plates were stored at 4°C overnight before washing five times with wash buffer; PBS plus 0·05 per cent Tween 80 (PBST). Test serum samples were diluted 1/100 in serum buffer (PBST plus 1 per cent skimmed milk powder, Premier Brands), added in 100 μl volumes to duplicate wells and the sealed plates incubated at 37°C for 90 minutes. Plates were washed as before and 100 μl donkey anti-sheep horseradish peroxidase (HRP) conjugate (Scottish Antibody Production Unit), diluted 1/200 in serum buffer added to all wells. Sealed plates were again incubated for 90 minutes at 37°C before washing as described. Finally 100 μl o substrate (4 mg ortho-phenylenediamine dihydro chloride plus 4 μl, 100 volume hydrogen peroxide ir 2·43 ml, 0·1 M citric acid; 2·57 ml, 0·2 M sodium hydrogen phosphate and 5 ml distilled water) wa added to all wells. After 15 minutes at room tem perature the reaction was stopped by the addition o 50 μl, 2·3 M sulphuric acid. The optical densities a 492 nm were determined on a Titertek plate reade (Flow Laboratories).

ELISA results were expressed as percentage optica density (OD) where:

$$\% \text{ OD} = \frac{(\text{mean OD test serum} - \text{blank})}{(\text{mean OD standard} - \text{blank})} \times 100.$$

Positive control was a convalescent serum used at a dilution of 1/100 in serum buffer, which consistentl gave OD values in the order of 2·5.

Whole cell ELISA

Heat-killed organisms at a concentration of 80 μg protein ml^{-1} in PBS were coated onto Dynatech M129A microtitre plates at 100 μl per well. Oper plates were dried overnight at 37°C and before use subjected to heating in a microwave oven (Philips Cooktronic 7910) for 10 minutes. Wells were blocked with PBST plus 4 per cent skimmed milk powder and left covered for 90 minutes at 37°C. Thereafter the procedure was as for the flagellin ELISA, except tha serum samples were tested at a fixed dilution of 1/400 in serum buffer.

SDS-PAGE

SDS-PAGE was performed by the method of Laemmli (1970), with a discontinuous system employing slab gels 0·75 mm thick, a 10 per cent (w/v) resolving ge and a 3 per cent (w/v) stacking gel. Samples were mixed 1:1 with sample buffer containing: 2 per cent SDS, 5 per cent 2-mercaptoethanol, 10 per cent sucrose, 0·01 per cent bromophenol blue, and boiled for three minutes before application to gels. Gels were loaded with a final volume of 200 μl of the cholestero precipitated listeriolysin O. Molecular weigh standards (Sigma MW SDS200) were also run on each gel.

Western blotting

After SDS-PAGE, proteins were transferred over night to Immobilon-P membranes (Millipore), at 0· A×40 V, using a mini-protean II immunoblo apparatus (BioRad). Molecular weight markers were stained with Ponceau red (Sigma) and their position noted before blocking. Blocking was by incubation

for one hour at 37°C in horse serum (Gibco) diluted 1:1 in wash buffer (0·35 M sodium chloride, 1 mM EDTA and 0·005 per cent Tween 20 in PBS). Test serum samples were diluted 1/100 in wash buffer plus 10 per cent horse serum. Hyperimmune anti-listeriolysin O rabbit serum (kindly provided by A. Haas, University of Wurzburg) was diluted 1/500 in the same diluent. Using mini-protean II multiscreen equipment (BioRad) serum samples were placed in rows on the blot membrane. Blots were incubated for two hours at 37°C, then thoroughly washed in wash buffer. Membranes were cut and incubated with the relevant conjugates for two hours at 37°C. Donkey anti-rabbit HRP, and donkey anti-sheep HRP (Scottish Antibody Production Unit) were used at dilutions of 1/200 in wash buffer plus 10 per cent horse serum. Then blots were thoroughly washed in wash buffer before a final rinse in 0·1 M Tris pH 7·4. Substrate (3 mg diaminobenzidine, plus 10 μl 100 volume hydrogen peroxide in 0·1 M Tris pH 7·4), was added and reactions were allowed to proceed for exactly 60 seconds before being stopped by washing the blots in tap water. To determine the sheep antibody subclasses involved mouse monoclonal antibodies recognising sheep IgG_1 and IgM subclass antibodies (kindly provided by Hoechst) were used as an intermediate step in the development of immunoblots. The conjugate was sheep anti-mouse HRP (Scottish Antibody Production Unit).

Animals

Twelve six-month-old Suffolk cross Blackface lambs were randomly allocated to two groups of six and housed separately. The lambs were born on the premises and had never been fed silage. Feeding consisted of hay and concentrate.

Experimental protocol

All the oral doses were deposited through tubing in the pharynx. Group 1 lambs received 5 ml PBS per os on three consecutive days. Group 2 lambs were given 1×10^{10} cell forming units (cfu) of *L monocytogenes* per day suspended in 5 ml PBS on the same days. Over the challenge period and for four days after, clinical examinations were made daily and rectal temperatures recorded.

The level of protective immunity conferred by oral challenge with viable *L monocytogenes* was assessed by comparing the clinical responses of both groups to a subcutaneous injection, given on the side of the animals' necks, of 2×10^{10} cfu of *L monocytogenes*. All lambs in groups 1 and 2 were challenged 24 days after the oral dosing. Following the subcutaneous challenge, clinical examinations were made and rectal temperatures recorded daily for seven days.

Blood samples were cultured on days 0 to 6 after the oral challenges and following the subcutaneous injections on days 0 to 4. Ten ml of venous blood were inoculated into Liquoid blood culture bottles (Roche) and these were incubated at 37°C for 48 hours. After 24 and 48 hours incubation subcultures were made onto sheep blood agar plates, which were incubated for 48 hours at 37°C and examined for bacterial growth. *L monocytogenes* was identified as previously described.

Serum was collected from blood samples taken on days 0, 7, 14 and 21 after the oral dosing and days 11, 25 and 39 after the subcutanous challenges. Sera were stored at −20°C before testing.

Pathological examinations

Full post mortem examinations were performed on two animals after the subcutaneous challenge. Samples of brain at the level of the medulla, plus liver, spleen and kidney were taken aseptically and inoculated onto 5 per cent sheep blood agar plates, incubated at 37°C and examined for bacterial growth over 72 hours. The samples of medulla were also macerated in 10 ml volumes of tryptose phosphate broth (Oxoid), stored at 4°C and subcultured weekly to 5 per cent sheep blood agar plates which were incubated and examined as described. The following tissues were collected into phosphate buffered, neutral 10 per cent formalin: brain, spinal cord, spleen, kidney and liver. Blocks of these tissues, 3 mm thick, were prepared, dehydrated in alcohol, embedded in wax, sectioned at 4 μm and stained with haematoxylin and eosin. Coronal slices of the brains were taken from the forebrain at the level of the optic tract, from the midbrain at the level of the lateral geniculate body, from the medulla at the level of the inferior cerebellar peduncle and from the anterior cervical spinal cord. Sagittal sections were taken from the cerebellum.

Statistical analysis

All statistical analysis was done using minitab statistical computer program package (State College, Pennsylvania, USA). Differrences between means were evaluated by Student's two sample *t* test. Results of blood culture for the two groups were tested by χ^2 analysis.

Results

Clinical findings

No clinical signs were observed in any lamb after the oral dosing, although the mean rectal temperatures of lambs in both groups were raised 24 hours after the first oral dose. At 48 hours the mean

TABLE 1: Mean rectal temperatures (°C) of lambs and isolation of *L monocytogenes* from blood samples after subcutaneous challenge with *L monocytogenes* 2×10^{10} cfu on day 0

	Control lambs (group 1) (n = 6)		Experimental lambs (group 2) (n = 6)	
	Mean rectal temperature ± SD	Positive culture/ number of blood cultures made	Mean rectal temperature ± SD	Positive culture/ number of blood cultures made
Day −1	39·7 ± 0·5	ND	40·0 ± 0·4	ND
Day 0	39·8 ± 0·7	0/6	40·1 ± 0·3	0/6
Day +1	40·8 ± 0·6	1/6	41·0 ± 0·5	0/6
Day 2	41·2 ± 0·5	5/6	40·1 ± 0·3*	0/6†
Day 3	40·3 ± 0·8	3/6	39·5 ± 0·5	0/6
Day 4	39·7 ± 0·4	1/6	39·7 ± 0·4	0/6
Day 5	39·8 ± 0·5	ND	39·8 ± 0·4	ND
Day 6	39·6 ± 0·3	ND	39·8 ± 0·4	ND
Day 7	39·5 ± 0·3	ND	39·6 ± 0·3	ND

* Significance in Student's *t* test $P < 0{\cdot}005$
† Significance in χ^2 analysis $P < 0{\cdot}01$
ND Not done

rectal temperature of group 2 lambs was significantly higher than that of group 1 (mean rectal temperatures ± standard deviation: group 2, 40·7 ± 0·3; group 1, 39·8 ± 0·5; $P < 0{\cdot}05$). At no other time before the subcutaneous injections were there significant differences in mean rectal temperatures of the two groups.

Lambs in both groups were pyrexic 24 hours after the subcutaneous injections. The rectal temperatures subsided to normal at 48 hours in the group 2 lambs but were elevated for 72 hours in the group 1 lambs. At 48 hours after challenge there was a significant difference between the two groups ($P < 0{\cdot}005$) (Table 1). The only discernible clinical signs of infection were increased respiratory rates for the duration of pyrexia. The lambs did not become inappetant or develop diarrhoea.

Forty-eight hours after the subcutaneous injections one lamb in group 2 (case 1) was unsteady and showed hindlimb incoordination when moved. The animal's head was not held to either side but was carried unusually low. The rectal temperature was 40·3°C. The clinical condition slowly improved over the following days and when killed 25 days later the lamb was clinically normal. A full post mortem examination was carried out. On day 10 after the subcutaneous challenge another lamb in group 2 (case 2) developed neurological signs. This animal when moved showed incoordination of the hindlimbs and a high-stepping hindleg gait. The rectal temperature was 40·9°C. Eight hours later the animal had opisthotonus, was recumbent and making paddling movements with its legs. It was killed and a post mortem examination performed.

Serological responses

The changes in antibody titres were broadly similar in both the serum agglutination tests (SAT) (Fig 1) and whole cell ELISA (Fig 2). The group 1 control lambs had no measurable antibody response to *L monocytogenes* until after the subcutaneous injection of

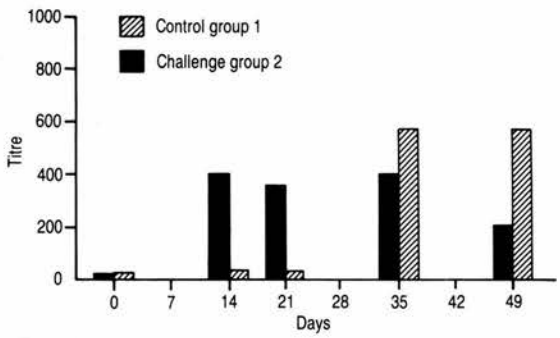

FIG 1: Geometric mean reciprocal titres in serum agglutination tests

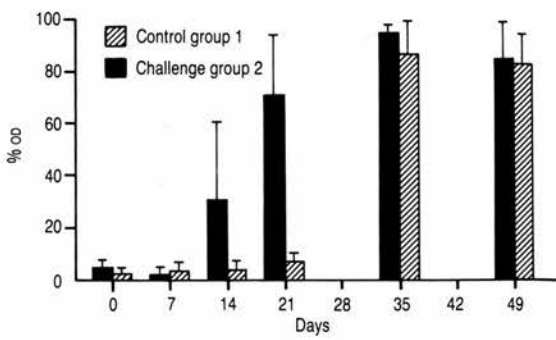

FIG 2: Serological responses of lambs to whole cell antigens as measured by ELISA

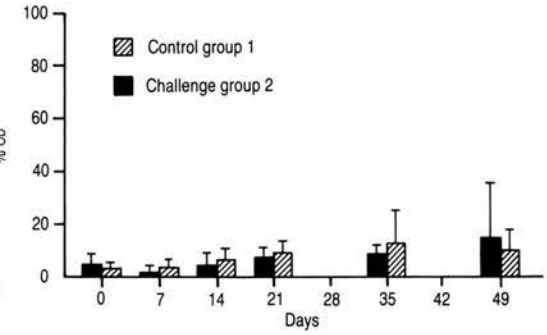

FIG 3: Serological responses of lambs to listeric flagellin as measured by ELISA

organisms when they attained titres comparable to group 2 lambs. By both ELISA and SAT, antibodies to listeric somatic antigens were detectable in the sera of group 2 lambs two weeks after oral challenge.

The antibody responses to purified flagellin following both oral and subcutaneous challenges were weak and variable (Fig 3).

The immunoblots of Fig 4 show examples of the serological responses to listeriolysin O. The group 2 lambs all developed antibody to this antigen following oral challenge whereas no antibodies were detectable in any prebleed or in the group 1 control lambs. Following the subcutaneous injections all the lambs had antibodies directed at listeriolysin O and these antibodies were maintained for at least 39 days. The subclass of antibody recognising listeriolysin was shown to be predominantly IgG_1, although a transient IgM response was identified with the group 1 control lambs after the subcutaneous challenges (Fig 5).

Bacteriological examinations

No *L monocytogenes* were cultured from blood samples taken in the six days after the oral challenge. Following the subcutaneous challenge *L monocytogenes* was never isolated from the blood of group 2 lambs, whereas blood cultures from five of the six lambs in group 1 were positive on 10 occasions over days 1 to 4 ($P < 0{\cdot}01$, Table 1).

After cold enrichment for one week *L monocytogenes* was isolated from the brain of case 2, which developed neurological symptoms on day 10 after the subcutaneous challenge. *L monocytogenes* was not isolated from any other tissue and from none of the tissues or brain of case 1 which was killed 25 days after the subcutaneous challenge.

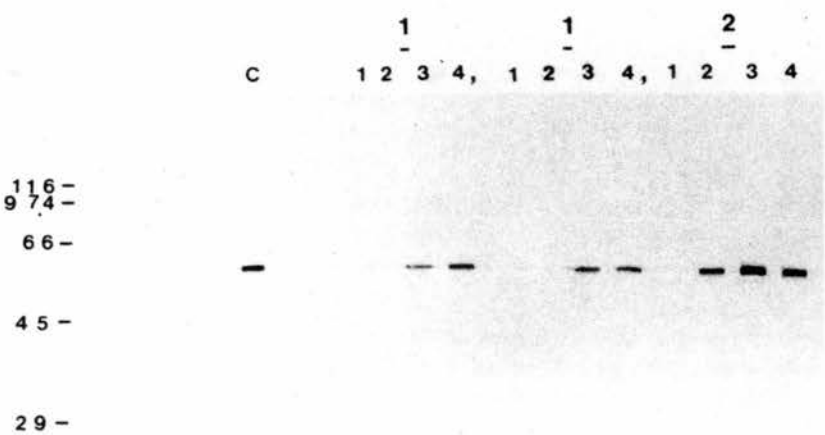

FIG 4: Serological responses of lambs to listeriolysin O demonstrated by immunoblotting. C Positive control rabbit antiserum, 1 Group 1 lambs, 2 Group 2 lambs, 1 Day 0, 2 Day 21, 3 Day 35, 4 Day 49. Positions of molecular weight markers (kDa) shown

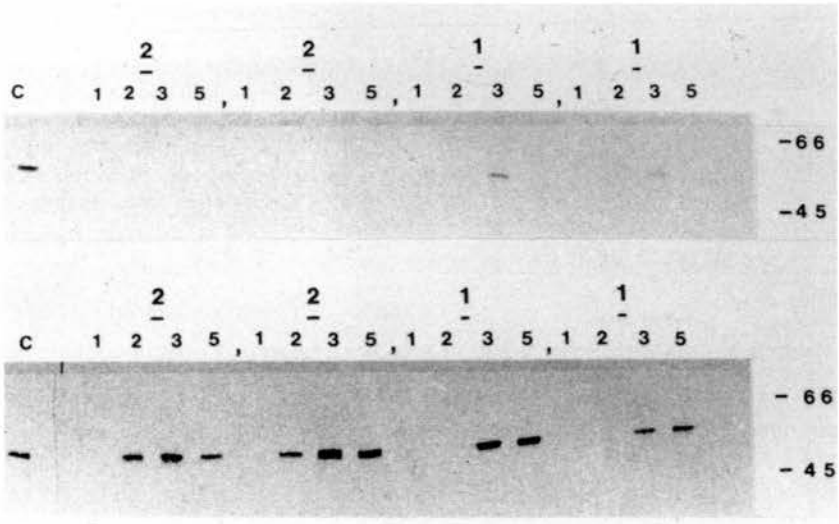

FIG 5: Subclass of antibody recognising listeriolysin O demonstrated by immunoblotting. (Above) IgM subclass response, (below) IgG_1 subclass response. C Positive control rabbit antiserum, 1 Group 1 lambs, 2 Group 2 lambs, 1 Day 0, 2 Day 21, 3 Day 35, 5 Day 63. Positions of molecular weight markers (kDa) shown

Pathological examinations

Post mortem examination revealed no gross lesions in either case 1 or 2 but histological lesions were considered to be characteristic for listeric encephalomyelitis. In case 1 there were unilateral microabscesses and lymphocytic perivascular cuffs in the pons, medulla and cervical spinal cord. A mild lymphocytic meningitis was also present. The microabscesses featured predominantly macrophage accumulation with local malacia, neuropil vacuolation and axonal degeneration. In the red nucleus of the midbrain, chromatolysis of cell bodies was found and in the cervical spinal cord areas of axonal swelling and wallerian degeneration were seen. No lesions were present in the other tissues examined. In case 2 the spinal cord was the principal site of the lesions. A single microabscess composed of macrophages and neutrophils was present in the cervical spinal cord and a mild radiculitis affected the ventral nerve root in the same section. A lymphocytic meningitis extended over the brain, and thick perivascular cuffs composed of lymphocytes were present in the spinal cord and the medulla, markedly unilateral in the latter. Extensive hepatocyte vacuolation consistent with fat accumulation was present in the liver, and in the kidney proteinaceous deposits were present in Bowman's capsules.

Discussion

Oral dosing of lambs with 3×10^{10} cfu of virulent *L monocytogenes* resulted in pyrexia at 48 hours but no apparent clinical illness. These animals seroconverted to listeric antigens and developed a degree of immunity to a subsequent subcutaneous challenge. The lack of a clinical response following the oral dosing is in agreement with Paterson (1940a) who produced no clinical signs in ewes given higher doses of the organism, and Graham et al (1940a) who recorded pyrexia of short duration following the oral dosing of a single lamb. Osebold and Inouye (1954) reported depression, inappetance and pyrexia in sheep given higher doses of the organism, though they comment that by cursory examination there was no apparent infection.

In this study the development of the humoral antibody response was found to be a consequence of challenge with live organisms since no increase in antibody titre occurred when lambs were dosed with a similar number of killed organisms (results not shown). The orally infected animals were shown to have developed a partial immunity to the subcutaneous challenge as there was a significant reduction in the number of positive blood cultures and significantly lower rectal temperatures. It is generally accepted that live organisms are necessary

for the production of immunity to listeric infection and that killed organisms are incapable of stimulating any protection (Graham et al 1940b, von Koenig and Finger 1982). It is possible that following the oral challenge systemic infection occurred even though no organisms were cultured from the blood. The occurrence of pyrexia at 48 hours after challenge supports this view, as this is within the period of greatest bacterial multiplication following oral infection (MacDonald and Carter 1980). It is suspected that the oral dosing of lambs resulted in multiplication of the organism with dissemination via the mesenteric lymph nodes to the liver, as has been shown to occur in mice and rats (MacDonald and Carter 1980, Schlech et al 1986). The failure to isolate the organism from the blood could be explained by its confinement to the lymphatic system and Kupffer's cells of the liver.

The primary objective of the work was the examination of antibody responses to listeric antigens and thus post mortem examinations of all the animals were not carried out at the end of the experiment. However, the development in two lambs of neurological signs together with histopathological lesions in the brain stems and spinal cords was intriguing, since neither lamb was found to be bacteraemic following the subcutaneous challenges, and upon culture *L monocytogenes* was isolated solely from the brain of case 2. Asahi et al (1957) suggested that infection of the brain arises via the cranial nerves. In this study there is circumstantial evidence based upon the topography of the lesions for infection of the central nervous system arising via the spinal nerves following the subcutaneous injections on the side of the neck. Since neurological symptoms occurred only in animals previously challenged per os there is tenuous support for the suggestion of Barley (1990) that listeric encephalitis is an immunopathological disease which arises in animals previously exposed to the organism. Both these aspects of the pathogenesis of listeric encephalitis merit further study.

The results of the whole cell ELISA and SAT are useful in monitoring the development of antibodies against listeric antigens. The serological responses measured by ELISA and SAT were in general agreement, though in one animal no agglutinins were detectable following the subcutaneous challenge. This animal did develop antibodies to whole organisms detectable in ELISA. The presence of low SAT titres in the prebleeds of these conventional lambs agrees with many of the reports reviewed by Seeliger (1961). However, owing to the use of crude, ill-defined antigens both assays lack specificity. The development of a useful serological test is dependent upon the identification of defined antigens and the examination of antibody responses to these antigens. In this study antibody responses to two candidate antigens, flagellin and listeriolysin O, were examined.

The antibody responses to flagellin were shown to be inconsistent. This is an intriguing result since 'flagellar' antigens have been part of the serological classification system of listeria for many years (Paterson 1940b) and have been used for serodiagnosis (Seeliger 1961). The antibodies measured in these assays might not be directed at flagellin but other proteins in the crude flagellar preparations, or they may be the result of exposure to bacteria expressing flagellin through growth at environmental temperatures. The work confirms the assertion of Peel (1987) that flagellin is not expressed 'in vivo' and is apparently not a useful antigen for serological or epidemiological studies.

The animals seroconverted to listeriolysin O after oral or subcutaneous infection and antibodies were still present when measured 39 days later. The results show that the predominant subclass of antibody produced against listeriolysin O is IgG_1. This is in agreement with findings in man (P. Berche, personal communication). These antibody responses are highly encouraging for the use of this antigen in immunoassays. As suggested by Berche et al (1990) detection of anti-listeriolysin O may be a reliable and useful method for epidemiological surveys and for diagnosis of listeriosis. Since listeriolysin O is produced by all pathogenic strains of *L monocytogenes* its use avoids the necessity of multiple serotype antigens in immunoassays.

A problem with cross reacting antibodies to listeriolysin O might have been expected as haemolysins produced by members of the genera *Streptococcus, Bacillus, Clostridium* and *Listeria* (Alouf and Geoffroy 1984) share antigenic relationships (Geoffroy and Alouf 1984, Geoffroy et al 1987). However, despite prior routine vaccination of the lambs against clostridial infections no evidence of cross reacting antibodies was found in these animals. The reason may be that the tertiary structure of the antigen had been destroyed in SDS-PAGE leaving only specific epitopes to react in immunoblotting. Further work is necessary to determine whether non-specific antibodies recognising listeriolysin O are prevalent in sheep.

This study has confirmed the findings of Berche et al (1990) that the detection of antibodies to listeriolysin O is useful for the serodiagnosis of listeriosis. Studies into the prevalence of antibodies against listeriolysin O in sera from field cases of listeriosis and flocks in which infection has occurred should be useful in improving our understanding of the epidemiology and pathogenesis of listeric infections in sheep.

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Purification of Listeriolysin O and Development of an Immunoassay for Diagnosis of Listeric Infections in Sheep

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A protein of 58,000-Da molecular mass was purified from the supernatant fluid of a dialysis sac culture of *Listeria monocytogenes* by cation-exchange chromatography. The purified protein, homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and possessing the characteristics of listeriolysin O (LLO), was used to develop an indirect enzyme-linked immunosorbent assay. Anti-LLO antibodies were shown to be consistently produced in sheep after experimental challenge with *L. monocytogenes* serovar 4b. The assay also successfully detected and measured specific anti-LLO antibodies in the sera of silage-fed sheep among which listeric enteritis and abortions had occurred.

Listeriosis is a major disease of sheep in the United Kingdom (7), but diagnosis and epidemiological investigations are severely restricted by the nonspecific and insensitive nature of the serological tests currently available (2, 8). Recently, listeriolysin O (LLO), a major virulence factor produced by all pathogenic strains of *Listeria monocytogenes*, has been identified as a candidate antigen for a serological assay (8). Antibodies to LLO were shown to be reliable indicators of experimental infections in immunoblotting studies (8, 11), and a dot blot assay based upon LLO has been developed for the diagnosis of listeric infections in humans (2).

LLO is antigenically related to other sulfhydryl-activated toxins produced by members of the genera *Streptococcus*, *Bacillus*, *Clostridium*, and *Listeria* (1, 4). However, antigenic variation and differences in gene sequence exist in streptolysin O (SLO), LLO, and pneumolysin (3, 10). The demonstration that anti-LLO antibodies could be detected in sera after absorption with SLO (2) is evidence of specific antigenic epitopes in LLO, and it is thus feasible that an immunoassay based upon purified LLO will be a reliable indicator of listeric infection. The development and application of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of specific anti-LLO antibodies in sheep is described.

MATERIALS AND METHODS

Bacterial culture. *L. monocytogenes* serovar 4b (strain L1059), isolated from a patient with listeric encephalitis, was stored at −70°C, and overnight growth was obtained by culture on 5% sheep blood agar plates. Dialysis sac cultures were produced according to the principles described by Sutherland (15).

Harvesting the supernatant fluid. The contents of the dialysis sac were collected and centrifuged at 4,300 × *g* for 45 min at 4°C, and the supernatant fluid was filtered through a 0.45-μm-pore-size Millipore filter. The filtrate was dialysed at 4°C against 5 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) adjusted to pH 6.5 with NaOH, and the 1-liter dialysis buffer volume was changed six times over 24 h. The dialyzed supernatant fluid (approximately 80 ml) was lyophilized and typically produced 280 mg of freeze-dried material from each dialysis sac.

Sulfopropyl (SP)-cation-exchange chromatography. The lyophilized supernatant fluid was rehydrated by addition of 1/10 the original volume of distilled water plus 5% (vol/vol) glycerol and 1 mM β-mercaptoethanol. A Glaspac TSK-SP-5PW high-performance liquid chromatography column (8 × 75 mm; Pharmacia LKB, Uppsala, Sweden) was equilibrated at room temperature with a buffer containing 50 mM MES, 1 mM β-mercaptoethanol, and 5% (vol/vol) glycerol, pH 6.5. The concentrated supernatant fluid was applied to the column, and after washing exhaustively the hemolysin was eluted with a 0 to 0.6 M NaCl gradient applied over 30 min at a flow rate of 0.5 ml/min. Fractions were collected every 2 min. The A_{280} and the hemolytic activity of column fractions were monitored. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (6), and the purity of LLO was assessed by silver staining (12) and immunoblotting (14).

Hemolysin assay. Hemolytic activity was determined according to the method of Kreft et al. (5). One hemolytic unit was defined as the reciprocal of the dilution which gave 50% hemolysis of a 1% (vol/vol) concentration of sheep erythrocytes.

Experimental sera. Details of the experimental challenge of lambs with *L. monocytogenes* serovar 4b have been described previously (8), and sera derived from the experiments were used in the development of the anti-LLO immunoassay.

Field sera. Twenty-four serum samples from four groups of sheep (A, B, C, and D) in a 500-ewe flock in which listeric infections were suspected were tested by ELISA. Six weeks after introduction to silage feeding, 14 ewes developed severe diarrhea (group A), and 5 days later eight abortions occurred (group B). *L. monocytogenes* serovar 1/2a was isolated upon the direct culture of fetal stomach contents and placentas of two abortion specimens. The majority of the ewes showed no clinical signs of infection (group C) and group D animals were only fed silage for 3 weeks prior to the outbreak. Blood samples were collected from six ewes in each group 2 days after the abortions occurred.

Determination by indirect ELISA of the optimal antigen dilution. A checkerboard titration was performed with serial dilutions of purified LLO in 0.1 M carbonate-bicarbonate

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buffer, pH 9.6, against doubling dilutions of a previously described convalescent antiserum (8). Plates were coated with antigen dilutions (100 μl per well) and were left sealed at 4°C overnight before continuation of the assay as described (8).

Testing of experimental and field sera for anti-LLO antibodies by indirect ELISA. Purified LLO was used at a concentration of 25 ng per well in 0.1 M carbonate-bicarbonate buffer, pH 9.6. Plates were coated and prepared as described above. Standard or test sera diluted in serum buffer were added to all wells except those in column 1, to which serum buffer alone was added. The test sera were diluted 1:400. The standard positive control was the convalescent antiserum made up in eight twofold dilutions from 1:100 to 1:12,800. The standard negative control serum was taken from a 6-month-old lamb and diluted 1:400 in serum buffer. Each sample was tested in duplicate, and plates were sealed and incubated at 37°C for 60 min. The assay was then continued as described (8).

Calculation of results. The A_{492} figures were converted to $\log_{10}$ values, and those for the standard serum were plotted against $\log_{10}$ values of the dilutions to provide a standard curve. The titer of the standard serum was taken to be that dilution which gave an A_{492} of 0.1, and the test serum titers were calculated from the formula: titer of test serum = [reciprocal of dilution of test serum (1:400)/reciprocal of standard antiserum dilution which would give the same absorbance value as the test serum] × titer of standard.

Testing of field sera for antibodies to LLO and SLO by immunoblotting. After electrophoresis in 10% polyacrylamide gels, samples of 5 μg of purified LLO or 5 μg of SLO (Sigma, Poole, England) were transferred to Immobilon P membranes. Seventeen of the field sera were tested for recognition of LLO and SLO in immunoblots using the technique described previously (8), with skim milk powder in place of horse serum in the blocking buffer and serum diluent. A horse anti-SLO serum (Wellcome Diagnostics, Dartford, England) and a hyperimmune anti-LLO rabbit serum (kindly provided by A. Haas, University of Wurzburg) were used as positive controls in the respective immunoblots.

RESULTS

Chromatography of the concentrated supernatant fluid on the SP-cation-exchange column produced two major peaks of absorbance. Hemolysin eluted reproducibly from the column at 0.21 to 0.28 M NaCl, pH 6.5, as the second major peak and was collected in fraction 13. This fraction retained a hemolytic activity of 640 hemolytic units and contained approximately 50 μg of protein per ml, representing approximately 20% recovery of lytic activity and a 120-fold purification. In SDS-PAGE, fraction 13 was shown by silver staining to contain a single homogeneous protein with a molecular mass of 58,000 Da (Fig. 1a). The protein was recognized in immunoblots both by the convalescent antiserum (Fig. 1b) and by a hyperimmune anti-LLO rabbit serum (Fig. 2). The addition of 0.1 mM free cholesterol to the hemolysin assay totally abrogated the hemolytic activity of the protein.

The optimal dilution of stock purified LLO for use in the indirect ELISA was established against a series of convalescent antiserum dilutions. Maximal optical density values were produced with antigen concentrations of 12.5 to 50 ng per well. When antigen coating at 25 ng per well was used,

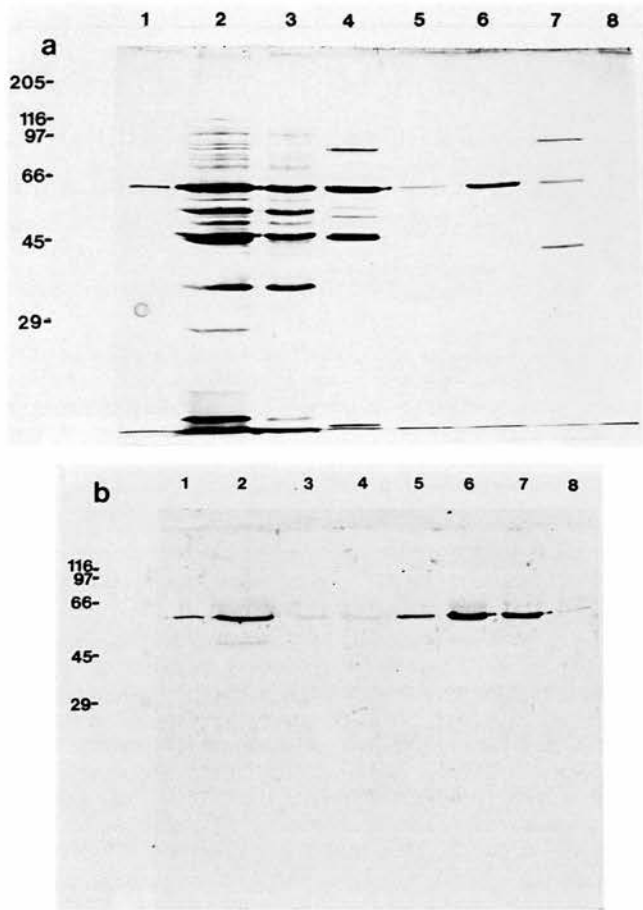

FIG. 1. (a) Silver staining of dialysis sac culture supernatant fluid and SP-cation-exchange fractions in SDS-PAGE; (b) immunoblot of dialysis sac culture supernatant fluid and SP-cation-exchange fractions, using convalescent antiserum. Lanes: 1, *L. monocytogenes* dialysis sac supernatant fluid (4 μg of total protein); 2, 10× dialysis sac supernatant fluid (32.5 μg of total protein); 3, SP-cation-exchange column flowthrough (15 μg of total protein); 4, fraction 10 collected from SP-cation-exchange column (0.5 μg of total protein); 5, fraction 12 (0.1 μg of total protein); 6, fraction 13 (0.5 μg of total protein); 7, fraction 14 (0.4 μg of total protein); 8, fraction 16 (0.2 μg of total protein). Positions of molecular mass markers (in kilodaltons) are indicated as numbers. Lane 6 contains approximately twice the amount of hemolysin as lane 2, and the purified protein migrates as a single band of M_r ca. 58,000 (lane 6).

the positive control sera produced A_{492} values in the range of 0.2 to 1.4 at serum dilutions of 1/200 to 1/800, and the log dose-response curve was linear for each antibody tested. For each serum, increasing antibody dilution was shown to have less effect on absorbance than decreasing the antigen coating.

On the basis of these results, the experimental sera were tested by indirect ELISA with antigen coating of 25 ng per well and using a 1:400 dilution of antibody. No recognition of LLO occurred with any of the sera taken prior to challenge. However, at 2 and 3 weeks after oral dosing with *L. monocytogenes*, low titers were detectable in five of the six experimental lambs. Serum titers of the control lambs remained <400 until after the subcutaneous challenges, when all animals had detectable antibody to LLO which was still present 4 weeks later (Table 1). In further experimental

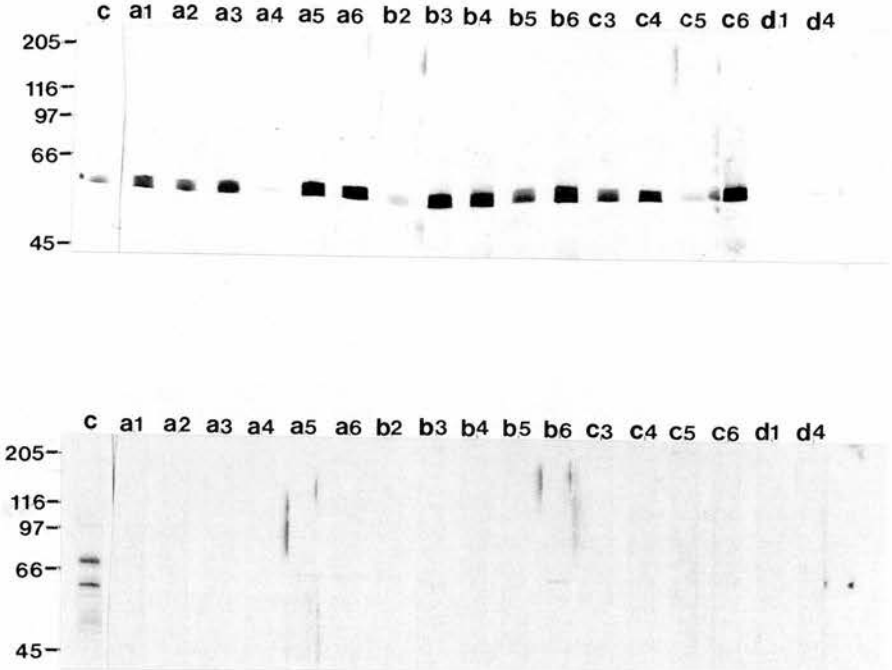

FIG. 2. Examination of field sera for recognition of LLO and SLO by immunoblotting. Position of molecular mass markers (in kilodaltons) are indicated as numbers. C indicates lanes probed with positive control antisera. Responses to LLO (top) and SLO (bottom) by 17 field serum samples are visible in the marked lanes. Group A ewes had severe diarrhea 7 days before sampling. Group B ewes had aborted two days before sampling. Group C ewes were silage fed but remained clinically normal. Group D ewes had only been fed silage for 3 weeks. The sera specifically recognized purified LLO, and the results of immunoblotting correlated with ELISA titers (Table 2).

studies the indirect ELISA has been shown to have a sensitivity of 82% for the discrimination of infected animals after oral challenge and a percentage agreement with immunoblotting of 94%.

Anti-LLO antibodies were demonstrated in the field sera tested by indirect ELISA (Table 2), and results were comparable to those obtained by immunoblotting (Fig. 2). Anti-LLO antibody titers similar to those seen after experimental challenge with *L. monocytogenes* were present in all animals in group A and five animals in group B. Antibodies to LLO were demonstrated in three animals in group C which had shown no clinical signs of infection. Finally for the animals in group D, which had been fed silage for only a short period, one animal had a low anti-LLO titer. No anti-SLO antibodies were detected in the field sera by immunoblotting (Fig. 2).

TABLE 1. Anti-LLO antibodies in experimental ovine sera determined by indirect ELISA

Day	Mean anti-LLO titer (± SE)	
	Experimental group[a] (n = 6)	Control group[b] (n = 6)
0	<400	<400
14	2,137 ± 1,243	<400
21	2,580 ± 1,021	<400
35	13,756 ± 1,501	7,715 ± 953
49	8,618 ± 1,448	8,666 ± 1,192

[a] Challenged orally with 1×10^{10} CFU of viable *L. monocytogenes* on days 0, 1, and 2 and injected subcutaneously with 2×10^{10} CFU on day 24.

[b] Dosed orally with phosphate-buffered saline on days 0, 1, and 2 and injected subcutaneously with 2×10^{10} CFU of viable *L. monocytogenes* on day 24.

DISCUSSION

The results demonstrate that a hemolytic protein possessing the characteristics of LLO (4, 5) was purified from the supernatant fluid of *L. monocytogenes* grown in dialysis sac culture. The hemolysin was shown in SDS-PAGE to be a single homogeneous protein with a molecular mass of approximately 58,000 Da which was recognized in immunoblots by an anti-LLO antiserum. The hemolytic activity could be abolished by small quantities of cholesterol.

The purification method for LLO was more efficient than the thiol-disulfide exchange methods previously published (4, 5), and considerable amounts of protein were obtained which remained antigenic and bound efficiently to polystyrene microtiter plates. Through the use of experimental sheep sera, antigen coating was optimized and an indirect

TABLE 2. Anti-LLO antibodies in field sera determined by indirect ELISA

Animal	Anti-LLO titer for group[a]:			
	A	B	C	D
1	3,571	<400	<400	<400
2	3,086	564	<400	<400
3	7,101	5,532	1,671	<400
4	510	12,396	4,809	510
5	10,062	3,376	<400	<400
6	3,331	12,240	10,861	<400

[a] Group A ewes had severe diarrhea 7 days before sampling; group B ewes had aborted 2 days before sampling; group C ewes were silage fed but remained clinically normal; group D ewes had only been fed silage for 3 weeks.

ELISA based upon LLO was developed. The assay confirmed previously published reports that seroconversion to LLO consistently occurred after oral or subcutaneous challenge with viable *L. monocytogenes* (8, 11).

The description of the field outbreak of disease is consistent with a previous report of septicemia and abortion caused by *L. monocytogenes* (9), and the ELISA confirmed the presence of anti-LLO antibody titers in two groups of ewes in which clinical disease had occurred. The presence of anti-LLO antibodies in three ewes from group C suggests that these animals were exposed to infection but remained clinically normal as is consistent with previous experimental findings (8). The ELISA titers in the field sera correlated with the results of immunoblotting using purified LLO as the antigen. The absence of antibodies which recognize SLO gives support to a previous report of specific antigenic epitopes in LLO (13). It appears from these results that the specific epitopes are also recognized during the course of natural listeric infections.

The low titer in a single ewe and absence of anti-LLO antibodies in the other ewes of group D and the prechallenge experimental sera are evidence that despite routine vaccination against clostridial disease cross-reaction with antibodies to clostridial toxins does not occur with ovine sera. The results demonstrate that the ELISA is a sensitive and reliable assay for the detection of anti-LLO antibodies.

The use of LLO in immunoassays thus avoids the necessity of multiple serotype antigens, and the described assay successfully measured specific anti-LLO antibodies in infected sheep. Application of the assay should help to improve our understanding of the epidemiology and pathogenesis of listeriosis in sheep.

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yrolysis mass spectrometry of *Listeria monocytogenes* olates from sheep

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rty-eight isolates of *Listeria monocytogenes* from eep and silage, involved in five small outbreaks listeriosis, were compared by pyrolysis mass ectrometry (PyMS). The method clustered isolates m single animals, and showed that epidemio- gically associated isolates were closely related each other. PyMS is a simple technique capable analysing large numbers of samples daily, and application in veterinary studies should help to cidate the epidemiology of listeriosis.

STERIA monocytogenes is widely distributed the environment, and listeriosis occurs world- de in a variety of animals including man eeliger 1961, Gray and Killinger 1966). Since e late 1970s the prevalence of infection appears have increased both in man (McLauchlin 1987) d sheep (Anon 1988a) and has become of con- erable significance. In sheep a number of con- ions are associated with infection, encephalitis d uterine infections being most common, ough septicaemia, iritis and other miscellaneous nditions also occur (Seeliger 1961, Gray and llinger 1966, Ladds et al 1974). Though recent vances in isolation techniques, and improve- nts in taxonomy and typing have extended r knowledge of listeria, epidemiological aspects infection remain poorly understood cLauchlin 1987). The preponderance in clini- disease of a limited number of serovars of *L nocytogenes* means that identification of epi- miologically related strains is impossible by otyping alone (Donker-Voet 1959, Seeliger and hne 1979, McLauchlin 1987) and alternative ing systems are urgently required (Anon 1988b). Phage-typing has not received wide application in the veterinary field since the majority of clinical isolates from sheep in the UK belong to serotype 1/2 (Audurier et al 1986) and cannot be consistently typed using this method (McLauchlin 1987). Other sophisticated techniques such as multilocus enzyme electrophoresis (Piffaretti et al 1989, Bibb et al 1990), restriction fragment length polymorphism (Saunders et al 1989) and restriction enzyme ana-lysis (Nocera et al 1990, Carriere et al 1991) are costly, time-consuming and available only in reference centres.

Recently, pyrolysis mass spectrometry (PyMS) has been shown to give useful, rapid interstrain comparisons within many bacterial species including *L monocytogenes* (Freeman et al 1990, 1991). In this paper, the PyMS characterisation of *L monocytogenes* isolates from diseased sheep and silage is reported.

Materials and methods

Bacterial isolates

The sources of 27 isolates of *L monocytogenes* from small outbreaks, designated O1 to O4, are shown in the table together with details of a further 21 isolates from two septicaemic sheep of one flock (designated O5).

Isolates were obtained from veterinary investigation centres throughout Scotland, were confirmed as *L monocytogenes* (Rocourt et al 1983) and serotyped at the Division of Microbiological Reagents and Quality Control, Colindale, London.

yrolysis mass spectrometry

Duplicate subcultures on fresh Diagnostic ensitivity Testing agar (Oxoid) plates of all isotes to be pyrolysed were incubated at 37°C vernight. Each subculture was regarded as a parate group and was sampled in triplicate nto pyrolysis foils (Horizon Instruments), dried 80°C for five minutes and pyrolysed on a PyMS 00X pyrolysis mass spectrometer (Horizon struments) using techniques and principles preously described (Freeman et al 1990, 1991). he resultant mass spectra were recorded on flopy disc together with the total ion count of each mple and its PyMS sequence number.

yMS data analysis

PyMS datasets of isolates from each outbreak ere created and analysed separately. The multiriate analysis of PyMS data has been described detail elsewhere (Freeman et al 1990, 1991). riefly, after normalisation to correct for variations sample size, the 30 mass ions giving the greatest etween group discrimination were subjected to rincipal component (pc) and canonical variate (cv) analyses. Interstrain comparisons were made by inspecting the ordination diagrams of PCCV1 versus PCCV2 using criteria documented previously (Freeman et al 1990). Isolates that could be distinguished from a cluster of strains were identified as outliers on the ordination diagram if their triplicate points did not overlap any from strains within the cluster. For clarity, these outlying strains which were 'distinct' from the remainder in the dataset were arbitrarily assigned numbers d1,d2, etc. Conversely, isolates in which the means of triplicate PyMS analyses of both subcultures closely abutted or overlapped each other on the ordination diagram were considered to be clustered by PyMS, and represented closely related isolates. Clusters of such isolates were arbitrarily assigned numbers c1,c2 etc.

Mahalanobis distances were used to construct similarity dendrograms clustered by the 'unweighted pair group method with averages' (UPGMA) (Sneath and Sokal 1973). Isolates that merged at a level above that at which duplicate subcultures of the same isolate did so were considered to be indistinguishable by PyMS.

Finally, representative isolates from all of the outbreaks were compared within one PyMS dataset.

BLE 1: The source of 48 isolates of *L monocytogenes* from five outbreaks of listeriosis in sheep

ease	Isolating laboratory	Isolate number †	Source of isolate	Serotype	PyMS result*
cephalitis	Edinburgh	O1-1, O1-2	Brain	4b	c1
	VIC (1991)	O1-3, O1-4	Brain	4b	c1
		O1-5 to O1-7	Brain	4 not 4b	c2
		O1-8	Silage	1/2a	d1
		O1-9	Silage	1/2b	d2
		O1-10	Silage	1/2a	d3
pticaemia	Inverness	O2-2, O2-3	Liver	1/2a	c3
	VIC (1991)	O2-1, 4, 5, 6	Liver	1/2a	c3
s	St Boswells	O3-1	Vaginal swab	1/2a	c4
	VIC (1990)	O3-2	Conjunctival swabs	1/2a	c4
		O3-3	Conjunctival swabs	1/2a	c4
		O3-4	Conjunctival swabs	1/2a	c4
		O3-5	Conjunctival swabs	1/2a	c4
		03-6	Conjunctival swabs	1/2a	c4
cephalitis	Edinburgh	O4-1	Brain	1/2a	c5
	VIC (1989)	O4-2	Brain	1/2a	c5
		O4-3	Brain	1/2a	c5
		O4-4	Brain	1/2a	c5
		O4-5	Brain	1/2a	c5
pticaemia	Edinburgh	O5-1, 2, 3, 4‡	Abomasum	1/2a	c6
	VIC (1990)	O5-5, 6, 7, 8	Faeces	1/2a	c6
		O5-9, 10, 11, 12	Abomasum	1/2a	c6
		O5-13, 14, 15, 16	Faeces	1/2a	c6
		O5-17, 18, 19, 20	Spleen	1/2a	c6
		O5-21	Brain	1/2a	c6

lusters of closely related isolates (c) and isolates that were clearly distinct from the remainder in the dataset (d) were arbitrarily ssigned numbers. Isolates with the same number, eg, c1, were part of the same cluster

Multiple isolates from a single source are separated by commas

solates O5-1 to O5-8 were obtained from one sheep; isolates O5-9 to O5-21 were obtained from a second sheep from the same flock

Results

Using the criteria for defining similarity/differences between isolates defined in the methods, the results of the PyMS analysis are shown in Table 1.

In encephalitis outbreak 01, the isolates of *L monocytogenes* from two sheep were indistinguishable (c1) but were quite distinct from the cluster of three isolates (c2) from another sheep in the same flock (Fig 1). Moreover, three isolates from silage bales were clearly differentiated from both these clusters and from each other (d1,d2,d3).

In the second outbreak (O2), six isolates from two septicaemic sheep were indistinguishable by PyMS (not shown). The six isolates of serovar 1/2a from vaginal and conjunctival swabs taken during a flock outbreak of abortion and iritis (O3) were indistinguishable by PyMS. Similarly, five further isolates of serovar 1/2a from another encephalitis outbreak (O4) were shown by PyMS, to be very closely related. The 21 isolates obtained from various tissues of two septicaemic sheep were indistinguishable by PyMS suggesting that they represented a single strain of *L monocyt genes*.

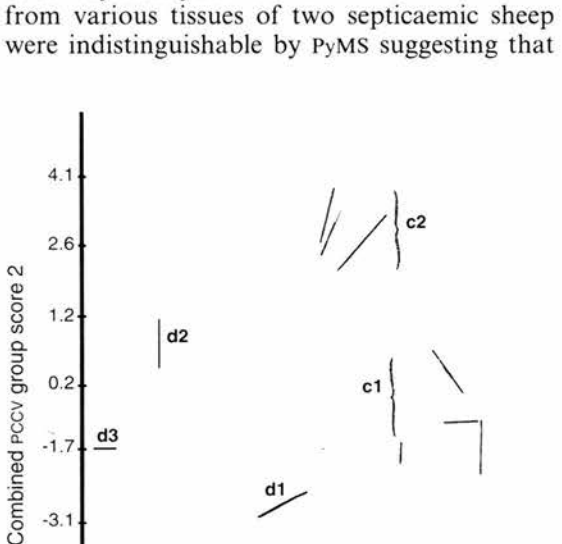

FIG 1: Ordination diagram for spectral data on duplicate subcultures of 10 isolates of *Listeria monocytogenes* from outbreak 1. The axes represent the first two canonical discriminant functions. The means of triplicate analyses of subcultures of the same isolate have been joined together

Comparison of representative isolates from of the outbreaks in a single PyMS dataset, show that the strain responsible for O2 was similar that causing O4, but could be distinguished fro the O3 strain. All were of serotype 1/2a, and th could not be differentiated by that metho Moreover, PyMS differentiated two strains serotype 1/2a isolated from silage.

Discussion

PyMS is a simple and relatively inexpensi technique capable of making rapid interstra comparisons (Freeman et al 1990) and its d criminatory ability for *L monocytogenes* h already been shown to be as accurate as curre genetic techniques (Freeman et al 199 Although PyMS does not assign permanent typ designations, its assessment of strain relatedne is valid for isolates pyrolysed as a single bat of freshly prepared media.

In this study, PyMS has clustered together mu tiple isolates of *L monocytogenes* taken fro single sheep, and has shown that isolates fro several diseased animals within a flock can very closely related. The demonstration of mo than one strain causing disease in the same flo is in agreement with previous phage-typing resu (Audurier et al 1986) and is an indication th during an outbreak animals may be exposed a number of different strains.

PyMS analysis has given results consistent wi the known, different origins of the strains studie and so the results are epidemiologically sensib despite the fact that many isolates were indisti guishable by serological typing. Conversely, t PyMS results showing that numerous isolates fro different organs in the same sheep are indisti guishable suggests that the method can detec strain of clonal origin.

None of the isolates of *L monocytogenes* fro silage was similar to clinical isolates from shee and the three strains from different silage ba in the outbreak O1 were distinguished from ea other by PyMS. Though only a limited numb of isolates from silage were available the resu suggest that silage may contain several differe strains which is in accord with previous phag typing studies (Nicolas et al 1988). Althou silage feeding has long been recognised as asso

ted with listeriosis (Gray 1960) it is important hat further studies are performed to determine he distribution and variety of *L monocytogenes* trains in silage and their relationship to isolates rom clinical disease.

Recent evidence has demonstrated that a single train of *L monocytogenes* serovar 4b has been esponsible for a number of foodborne outbreaks f listeriosis in man (Piffaretti et al 1989, Bibb t al 1990, Nocera et al 1990). In this study PyMS nalysis has identified the involvement of closely elated serovar 1/2a strains in two unrelated flock utbreaks of listeriosis, and has clustered together solates from individual outbreaks. It is therefore mportant that further veterinary isolates are xamined by PyMS and other discriminatory techiques such as multilocus enzyme electrophoresis nd restriction fragment length polymorphism o determine if these strains are particularly comnon and of major epidemiological importance.

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Hypothalamic-pituitary-adrenal axis responsiveness to insulin-induced hypoglycaemia is modified by trypanosome infection in Boran (*Bos indicus*) cattle

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Ten Boran (*Bos indicus*) cattle were used to study the stress responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis during trypanosome infection. Five cattle were infected with *Trypanosoma congolense* IL 1180 by tsetse challenge and five cattle served as controls. All infected animals developed acute trypanosomiasis. Insulin-induced hypoglycaemia (50 per cent of pre-insulin glucose concentration) was used as a stress factor. Acute hypoglycaemia was observed in three infected and three control animals after insulin challenge. Two animals from each group either did not respond or responded slowly. Hypoglycaemia in infected animals completely failed to induce an HPA axis response, while in control animals an HPA axis response was indicated by a significant increase in plasma adrenocorticotrophic hormone (ACTH) and cortisol concentrations ($P<0.01$). The results show that trypanosomiasis in Boran cattle can cause a decrease in the stress responsiveness of the HPA axis as indicated by a blunted ACTH/cortisol response to insulin-induced hypoglycaemia.

THE hypothalamic-pituitary-adrenal axis (HPA) is the endocrine pathway which is responsible for initiation of many stress reactions in the body. Stress responses have generally been demonstrated to increase the production of adrenocorticotrophic hormone (ACTH) and beta-endorphin by the pituitary under the control of the hypothalamic hormones corticotrophin releasing factor (CRF), oxytocin and vasopressin, resulting in an increased secretion of glucocorticoids by the adrenal cortex (Jones and Gillam 1988). As a consequence of these increases, circulating leve of glucocorticoids have been used as an indicat of intensity of stress response in animals (Corsell and De Rosa 1986).

The HPA axis is sensitive to stimuli such a trauma, noise, fear, fever and shock hypogly caemia, and the production of CRF, oxytocin an vasopressin by hypothalamic neurons is cor trolled by inputs to the hypothalamus from th limbic systems induced by the above stimuli.

The benefit of the stress reaction in the bod primarily comes from increased glucocorticoi secretion which initiates shifts in carbohydra metabolism, increasing circulating energy sub strates at the cost of energy stores. In the sho term, glucocorticoids limit immune and inflam matory reactions to infections, thus minimisin the cellular and tissue damage that they ma produce.

Experimentation by administration of a num ber of stressful stimuli, such as insulin-induce hypoglycaemia, vasopressin and pyrogens ha been developed to test the integrity of the HP (Corsello and De Rosa 1986). These stimuli, ac ing on hypothalamic and, or, pituitary level cause an increase in ACTH secretion so that b measuring plasma glucocorticoids, the status the axis can be evaluated.

The interaction of stress reactions and infe tious diseases has long been recognised. Anima suffering from trypanosomiasis are often cor sidered to be less tolerant to stress factors suc as work load, nutritional factors, manageme and vaccination interventions. Although the stress intolerances are also common in other di ease situations, in most cases they are considere

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"Did he smile his work to see?

Did he who made the Lamb make thee?"

William Blake.